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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C07H 21/04, 21/02, C12N 15/12, 15/70, 1/21</b>		<b>A1</b>	(11) International Publication Number: <b>WO 97/11956</b>
			(43) International Publication Date: <b>3 April 1997 (03.04.97)</b>
(21) International Application Number: <b>PCT/US96/15727</b>			(81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(22) International Filing Date: <b>27 September 1996 (27.09.96)</b>			
(30) Priority Data: <b>60/004,387 27 September 1995 (27.09.95) US</b>			
(60) Parent Application or Grant (63) Related by Continuation <b>US 60/004,387 (CIP) Filed on 27 September 1995 (27.09.95)</b>			
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(54) Title: <b>IDENTIFICATION OF sel-12 AND USES THEREOF</b>			
(57) Abstract  This invention provides an isolated nucleic acid molecule encoding a SEL-12. This invention further provides an isolated nucleic acid molecule which encodes a mutated SEL-12. This invention also provides an isolated nucleic acid molecule which encodes a mutated SEL-12, wherein the mutated SEL-12 contains at least one of the following: position 115 is a leucine, position 132 is an arginine, position 215 is a glutamic acid, position 229 is a valine, position 254 is a valine, position 255 is a valine, position 371 is a valine, position 387 is a tyrosine, position 104 is an isoleucine or position 204 is a valine. This invention further provides different uses of these nucleic acid molecules. This invention also provides different sel-12 mutants and transgenic animals which carry wild-type or mutated sel-12.			

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### IDENTIFICATION OF *sel-12* AND USES THEREOF

This application claims benefit of U.S. Provisional Application  
5 No. 60/004,387, filed September 27, 1995, the content of which  
is incorporated into this application by reference.

Within this application, publications are referenced within  
parentheses. Full citations for these references may be found  
10 at the end of each series of experiments. The disclosures of  
these publications in their entireties are hereby incorporated  
by reference into this application in order to more fully  
describe the state of the art to which this invention pertains.

#### 15 Background of the Invention

The *lin-12* gene of *C. elegans* is the archetype of the "*lin-12/Notch*" gene family found throughout the animal kingdom (reviewed in Greenwald and Rubin, 1992). Members of this family appear to function as receptors for intercellular signals that  
20 specify cell fates during development. Essentially, *lin-12* activity controls binary decisions: if a cell has a choice between two fates, A and B, activation of *lin-12* above a threshold value causes the cell to adopt fate A, whereas the failure to activate *lin-12* above the threshold causes the cell  
25 to adopt fate B (Greenwald et al. 1983). Furthermore, inappropriate activation of mammalian *lin-12/Notch* genes have been implicated in oncogenesis (Ellisen et al., 1991; Robbins et al., 1993) and in normal development (e.g. Swiatek et al., 1993). Much of the work in applicants' laboratory is focused  
30 on understanding how *lin-12* specifies cell fates. An important component of this endeavor is the identification of genes that influence *lin-12* activity and the identification of potential "downstream" genes.

35 Applicants identified the *sel-12* gene by screening for suppressors of the "Multivulva" phenotype caused by an allele of *lin-12* that causes constitutive LIN-12 activation. Applicants performed a genetic and molecular characterization of *sel-12*, which established: (1) Reducing or eliminating  
40 *sel-12* activity reduces the activity of *lin-12* and of *glp-1*, another member of the *lin-12/Notch* family. In addition,

- reducing or eliminating *sel-12* activity causes an egg-laying defective (Egl) phenotype. Applicants do not know if the Egl phenotype is a direct consequence of reducing *lin-12* activity or an independent effect of reducing *sel-12* activity. (2) *sel-12* and *lin-12* can functionally interact within the same cell. (3) *sel-12* is predicted to encode a protein with multiple transmembrane domains that is highly similar to S182, which has been implicated in early-onset familial Alzheimer's disease (Sherrington et al., 1995). These findings have been described in a paper that has been accepted by Nature (Levitan and Greenwald, 1995). In addition, applicants have data indicating that *sel-12* is more broadly expressed than *lin-12*, including a lot of expression in neurons.
- 15 The remarkable conservation of the SEL-12 and S182 predicted protein structure suggests that their functions are likely to be conserved as well. Recently, a second gene known as E5-1 or STM2 has been implicated in early-onset familial Alzheimer's disease (Levy-Lahad et al, 1995; Rogaev et al, 1995) E5-1/STM2 encodes a protein that is highly similar to S182 (Levy-Lahad et al, 1995b; Rogaev et al, 1995) and SEL-12. Furthermore, it is striking that four of the five changes in S182 or E5-1/STM2 associated with early-onset familial Alzheimer's disease alter amino acids that are absolutely conserved in the worm and the human proteins, and that the tenth alters an amino acid that has been changed very conservatively during evolution. Applicants hope to bring the powerful tools of classical and molecular genetic studies in *C. elegans* to bear on fundamental issues of SEL-12/S182/E5-1 structure and function. Thus, far, proteins similar to LIN-12 and SEL-12 have not been described in single-celled organisms, so *C. elegans* may be the simplest practical system for studying these issues in vivo.



Summary of the Invention

This invention provides an isolated nucleic acid molecule encoding a SEL-12 protein. This invention further provides an isolated nucleic acid molecule which encodes a mutated SEL-12  
5 protein. This invention also provides an isolated nucleic acid molecule which encodes a mutated SEL-12, wherein the mutated SEL-12 contains at least one of the following: position 115 is a leucine, position 132 is an arginine, position 215 is a glutamic acid, position 229 is a valine, position 254 is a  
10 valine, position 255 is a valine, position 371 is a valine, position 387 is tyrosine, position 104 is an isoleucine or position 204 is a valine. This invention further provides different uses of these nucleic acid molecules. This invention also provides different sel-12 mutants and transgenic animals  
15 which carry wild-type or mutated sel-12.

Brief Description of the Figures

Figure 1:

5 A. Nucleotide sequence and the deduced amino acid sequence of the *sel-12* cDNA. The first 22 nucleotides, shown in *italics*, correspond to the sequence of the trans-spliced leader SL1, a sequence found on the 5' end of many *C. elegans* transcripts 26. Potential membrane-spanning domains are underlined. No potential signal sequence was identified. Analysis of the amino acid sequence using the Kyte-Doolittle algorithm predicts that all nine domains have high enough hydrophobicity values to span a membrane. Three potential glycosylation sites (N-X-T/S) in the region between the seventh and eighth putative transmembrane domains are shown in *italics* at positions 273, 286, and 319 of the amino acid sequence. The locations of the introns are indicated by a caret over the nucleotide preceding the intron. *sel-12* contains seven exons and six introns and spans 2.3 kb of genomic DNA.

25 B. Schematic representation of the SEL-12 protein and molecular lesions associated with three *sel-12* alleles. Filled rectangles indicate nine hydrophobic regions. Based on the Kyte-Doolittle algorithm, they are potential membrane spanning domains. The fifth hydrophobic region contains only 18 amino acids and the sixth hydrophobic region contains a charged residue; however, these features are conserved in S182, so applicants infer that they are likely to be *bona fide* membrane-spanning domains. The ninth hydrophobic domain is not followed by a basic amino acid and is not conserved in S182 (although the C-terminus of S182 is relatively hydrophobic), so the inference that it is a membrane-spanning domain is more tentative. No potential signal sequence

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was identified.

Figure 2:

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Predicted protein sequence of SEL-12 and its alignment with the predicted protein sequences of S182 and E5-1/STM2. The Pileup program of the GCG-Wisconsin package was used to create this alignment. Amino acids that are identical between SEL-12 and one or more of the other proteins are highlighted in black, and predicted transmembrane domains are overlined. S182 is the predicted protein of a gene associate with early-onset familial Alzheimer's disease (Sherrington et al., 1995). E5-STM2 has also been implicated in early-onset familial Alzheimer's disease (Levy-Lahad et al., 1995a,b; Rogaev et al., 1995). The positions of the ten mutations associated with disease in S182 and E5-1/STM2 (Levy-Lahad et al., 1995b; Rogaev et al., 1995; Sherrington et al., 1995) are indicated (X), and tabulated in Table 1 below. SEL-12 and S182 are 48% identical, SEL-12 and E5-1/STM2 are 51% identical, and S182 and E-51/STM2 are 67% identical (Levy-Lahad et al., 1995b; Rogaev et al., 1995). SPE-4 is the predicated protein of the spe-4 gene of *C. elegans*, which is required for spermatogenesis (L'Hernault and Arduengo, 1992). SEL-12, S182 and E5-1/STM2 appear to be much more closely related to each other than they are to SPE-4. For example, S182 and SPE-4 are only 22% identical, with several large gaps. Furthermore, several regions that are very highly conserved between SEL-12, S182 and E5-1/STM2 are not conserved in SPE-4, and only one of the ten mutations associated with Alzheimer's disease affects an amino acid that is identical in SPE-4.

Figure 3.

Transgenic hermaphrodites expressing a

*sel-12::lacZ* transgene. Expression is seen in neural and non-neural cells. A. Adult. Large arrow indicates nerve ring; smaller arrows indicate muscle nuclei. B. Adult. Arrows indicate ventral cord nuclei. C. L3 larva. Arrows indicate nuclei of the vulval precursor cells P3.p-P8.p. D. L2 larva. Arrows indicate the nuclei of the somatic gonadal cells Z1.ppp and Z4.aaa. *sel-12* activity has been shown to influence the fates of P3.p-P8.p, and Z1.ppp and Z4.aaa in sensitized genetic backgrounds (11 of the Third Series of Experiments). Compromised neural function associated with reduced activity has not yet been seen in the nerve ring or ventral cord, possibly because an appropriate sensitized genetic background has not been examined. Complete genotype: *smg-1(r861) unc-54(r293); arIs17 [pRF4, pIB1Z17]*.

Detailed Description of the Invention

- This invention provides an isolated nucleic acid molecule encoding a SEL-12. This invention further provides an isolated nucleic acid molecule which encodes a mutated SEL-12.
- 5 This invention also provides an isolated nucleic acid molecule which encodes a mutated SEL-12, wherein the mutated SEL-12 contains at least one of the following: position 115 is a leucine, position 132 is an arginine, position 215 is a glutamic acid, position 229 is a valine, position 254 is a
- 10 valine, position 255 is a valine, position 371 is a valine, position 387 is tyrosine, position 104 is an isoleucine or position 204 is a valine. In an embodiment, the mutation is generated by in vitro mutagenesis.
- 15 In an embodiment, the isolated nucleic acid molecule is a DNA molecule. In a further embodiment, the DNA is a cDNA molecule. In another further embodiment, the DNA is a genomic DNA molecule. In a separate embodiment, the nucleic acid molecule is an isolated RNA molecule.
- 20 This invention also provides the above nucleic acid molecule which encodes substantially the same amino acid sequence as shown in Figure 1A.
- 25 This invention also provides a nucleic acid molecule of at least 15 nucleotide capable of specifically hybridizing with a unique sequence within the sequence of a nucleic acid molecule described above. In an embodiment, these nucleotide are DNA. In another embodiment, these nucleotide are RNA.
- 30 This invention also provides a vector which comprises the above-described isolated nucleic acid molecule. This invention also provides the above-described isolated nucleic acid molecules operatively linked to a promoter of RNA
- 35 transcription.

In an embodiment, the vector is a plasmid. In an embodiment, the Sel-12 genomic DNA, a MunI/XhoI genomic fragment was cloned into the Bluescript KS<sup>+</sup> plasmid which was cut with EcoRI and

XhoI. The resulting plasmid is designated as pMX8.

This plasmid, pMX8 was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. on September 14, 1995 under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The pMX8 was accorded with ATCC Accession number 97278.

10 In another embodiment, a Sel-12 cDNA, an EcoRI cDNA fragment was cloned into the Bluescript KS' plasmid which is cut with EcoRI. The resulting plasmid is designated p1-1E. The plasmid, p1-1E was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. 15 on September 14, 1995 under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. The p1-1E was accorded with ATCC Accession number 97279. This plasmid p1-1E containing a frameshift mutation in the 3' end of the 20 coding region of the cDNA. It can be easily corrected to the wild-type sequence as the complete sequence of *Caenorhabditis elegans* has been known.

This invention also provides a host vector system for the 25 production of a polypeptide having the biological activity of a SEL-12 or a mutated SEL-12 which comprises the above-described vector and a suitable host. The suitable hosts include but are not limited to bacterial cells, insect cells, plant and mammalian cells.

30 This invention also provides purified SEL-12 and mutated SEL-12.

This invention also provides a purified SEL-12 protein or a 35 purified SEL-12 fragment thereof. This invention further provides a purified mutated SEL-12 protein or a purified mutated SEL-12 fragment thereof.

This invention provides a method for production of an antibody

capable of binding to wild-type and/or mutant S182 or E5-1/STM2 comprising: a) administering an amount of the purified protein or fragment of SEL-12 or mutated SEL-12 to a suitable animal effective to produce an antibody against SEL-12 or mutated SEL-12 protein in the animal; and b) testing the produced antibody for capability to bind wild-type and/or mutant S182 or E5-1/STM2.

In an embodiment, the antibody is produced by in vitro immunization. In another embodiment, the antibody is produced by screening a differential phage display library. The produced antibody may be tested by Western blot analysis, immunoprecipitation, staining of cells or tissue sections or in combination of the above.

This invention also provides a method for production of an antibody capable of binding to wild-type and/or mutant S182 or E5-1/STM2 comprising: a) determining conserved regions revealed by alignment of the SEL-12, S182 and E5-1/STM2 protein sequences; b) synthesizing peptides corresponding to the revealed conserved regions; c) administering an amount of the synthesized peptides to a suitable animal effective to produce an antibody against the peptides in the animal; and b) testing the produced antibody for capability to bind wild-type and/or mutant S182 or E5-1/STM2.

In an embodiment, the antibody is produced by in vitro immunization. In another embodiment, the antibody is produced by screening a differential phage display library. The produced antibody may be tested by Western blot analysis, immunoprecipitation, staining of cells or tissue sections or in combination of the above.

This invention provides antibodies produced by above methods. This invention intends to cover other methods of production of antibodies capable of binding to wild-type and/or mutant S182 or E5-1/STM2 using the SEL-12 protein or sel-12. This invention also provides monoclonal antibodies capable of binding to wild-type and/or mutant S182 or E5-1/STM2.

This invention also provides antibodies capable of specifically recognizing SEL-12 protein or mutated SEL-12 protein. As used herein the term "specifically recognizing" means that the antibodies are capable of distinguish SEL-12 protein or mutated  
5 SEL-12 proteins from other proteins.

This invention also provides transgenic animals which express the above nucleic acid molecules. In an embodiment, the animal is a *Caenorhabditis elegans*. This invention also  
10 provides transgenic *Caenorhabditis elegans* animals comprising wild-type or mutant human S182 gene. This invention further provides transgenic *Caenorhabditis elegans* animals comprising wild-type or mutant human STM2/E5-1 gene.

15 This invention provides the above transgenic *Caenorhabditis elegans* animals, wherein the wild-type or mutant human S182, or wild-type or mutant STM2/E5-1 gene is under the control of *sel-12* or *lin-12* regulatory sequence.

20 This invention also provides a method for identifying a compound which is capable of ameliorating Alzheimer disease comprising administering effective amount of the compound to the transgenic animals or *sel-12* mutants, the alteration of the conditions of the transgenic animal indicating the compound is  
25 capable of ameliorating Alzheimer disease.

This invention also provides a previously unknown compound identified by the above method. This invention provides a pharmaceutical composition comprising an effective amount of  
30 the compound identified by the above method and a pharmaceutically acceptable carrier.

Pharmaceutically acceptable carriers are well known to those skilled in the art. Such pharmaceutically acceptable carriers  
35 may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions



or suspensions, saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, 5 electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, chelating agents, inert gases and the like.

10 This invention further provides a method for determining whether a compound might be capable of ameliorating Alzheimer's disease comprising: a) treating *Caenorhabditis elegans* mutants having reduced, increased or altered *sel-12* activity with the compound; and b) determining whether the compound suppresses, 15 enhances or has no effect on the phenotype of the mutant, the suppression or enhancement of the phenotype indicating the compound is capable of ameliorating Alzheimer's disease.

This invention provides a pharmaceutical composition comprising 20 an effective amount of the compound determined by the above method to be capable of ameliorating Alzheimer's disease and a pharmaceutically acceptable carrier.

This invention provides a method for identifying a suppressor 25 of the multivulva phenotype of *lin-12* gain-of-function mutation comprising: a) mutagenizing *lin-12 Caenorhabditis elegans* worms with an effective amount of an appropriate mutagen; b) screening for revertants in the F1, F2 and F3 generations; and c) isolating the screened revertant, thereby identifying a 30 suppressor of the multivulva phenotype of *lin-12*. This invention also provides suppressors identified by the above method.

In an embodiment, this invention provides a *Caenorhabditis elegans* animal having a suppressor, designated *sel-12(ar131)*. 35 This nematode was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. on September 27, 1995 under the provisions of the Budapest Treaty for the International Recognition of the

Deposit of Microorganism for the Purposes of Patent Procedure. *sel-12(ar131)* was accorded with ATCC Accession number 97293. In another embodiment, this invention provides an animal having a suppressor designated *sel-12(ar133)*.

5

This invention also provides a method for identifying a mutant *sel-12* gene which reduces *sel-12* function comprising:

- a) mutagenizing *Caenorhabditis elegans* worms with an effective amount of an appropriate mutagen; b) performing complementation screening of the mutagenized worms to determine if a descendant of a mutagenized worm bears a mutation that fails to complement one of the above-described suppressor for the *Egl* defect; and c) isolating the individual worm and determining the phenotype of worms carrying the new allele in its homozygous form and in trans to a deficiency, thereby identifying a mutant *sel-12* gene which reduces *sel-12* function. In an embodiment, this invention provides the above method which further comprises performing DNA sequence analysis of the identified mutant *sel-12* gene to determine the molecular lesion responsible for the mutation.

This invention also provides mutant *sel-12* genes identified by the above methods. In an embodiment, this invention provides an animal having a mutant *sel-12* gene, designated *sel-12(ar171)*. This nematode was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. on September 27, 1995 under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. *sel-12(ar171)* was accorded with ATCC Accession number 97292.

This invention provides a method for producing extragenic suppressors of a *sel-12* allele comprising: a) mutagenizing *sel-12* mutant hermaphrodites with an effective amount of a mutagen; b) screening for revertants in the F1, F2 and F3 generations; and c) isolating the screened revertant.

This invention also provides a method for producing extragenic

suppressors of a *sel-12*(Alz) mutant comprising: a) mutagenizing *sel-12* (Alz) hermaphrodites with an effective amount of a mutagen; b) screening for revertants in the F1, F2 and F3 generations; and c) isolating the screened revertant.

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Appropriate mutagens which may be used in this invention are well known in the art. In an embodiment, the mutagen is ethyl methanesulfonate.

- 10 This invention also provides suppressors produced by the above methods. This invention further provides a method for identification of a suppressor gene comprising performing DNA sequence analysis of the above suppressors to identify the suppressor gene. This invention also provides the identified  
15 suppressor gene by the above method.

This invention will be better understood from the examples which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are  
20 merely illustrative of the invention as described more fully in the claims which follow thereafter.

## Experimental Details

### First Series of Experiments

#### Materials and Methods

5 Applicants genetically mapped *sel-12* to the left of *unc-1 X*:  
from hermaphrodites of genotype *sel-12(ar131) dpy-3(e27)/unc-1(e538)*, 1/36 *Sel* non-*Dpy* and 18/19 *Dpy* non-*Sel* recombinants  
segregated *unc-1*. To clone *sel-12*, applicants used the well  
correlated genetic and physical maps in the *sel-12* region to  
10 identify cosmid clones that potentially carried the *sel-12* gene  
(ref. 27 and A. Coulson et al., personal communication). Applicants assayed pools and single cosmids for the ability to  
rescue the *Egl* defect of *sel-12 (ar131)* hermaphrodites, using  
the plasmid pRF4 [*rol-6 (su1006)*] as a dominant  
15 cotransformation marker (28). Ultimately, applicants found  
that pSpX4, containing a 3.5 kb *SpeI*//*Xho I* subclone of C08A12  
(Subcloned into KS Bluescript, Stratagene) completely rescue  
*sel-12(ar131)*. When this subclone was microinjected at a  
concentration of 10 µg/ml into *sel-12(ar131)* animals, 6/6 lines  
20 all demonstrated rescue of the *Egl* phenotype. When applicants  
attempted to obtain transgenic lines carrying pSpX4 using a  
concentration of 50 µg/ml, applicants obtained F1 transformants  
but no stable lines perhaps indicating some toxicity of this  
plasmid at higher concentrations. Applicants used this genomic  
25 subclone to screen a cDNA library (kindly provided by Bob  
Barstead) and identified one class of clones of 1.5 kb in size.  
All subcloning, restriction digests, and library screening were  
done according to standard techniques (29). Applicants  
sequenced both strands of the cDNA clone after generating  
30 systematic deletions using the Erase-a-base system (Promega®).  
DNA sequence was performed on double stranded templates using  
Sequenase (US Biochemical). The cDNA contained both a poly (A)  
tail and a portion of the spliced leader sequence SL1 (ref.  
30), suggesting it was a full length clone. Applicants  
35 confirmed the 5' end of the cDNA by RT-PCR (31). The sequence  
of this full-length cDNA can be found through GenBank under  
accession number U35660.

To identify the lesions associated with *sel-12* alleles

applicants used PCR to amplify the *sel-12* genomic fragment from DNA isolated from the *sel-12* mutant strains using the primers DL103 (5'TGTCTGAGTTACTAGTTTTCC 3')(SEQ. ID. 7) and DLG3 (5'GGAATCTGAAGCACCTGTAAGCAT 3')(SEQ. ID. 8). An aliquot of this double-stranded amplification product was used as the template in a subsequent round of PCR using only the primer DL103, to generate a single-stranded template. Exon specific primers were used to determine the entire coding sequence for all three alleles. For each allele, only one alteration in sequence was identified.

#### Experimental Result and Discussion

The *lin-12(d)* hypermorphic mutation *lin-12(n950)* causes a Multivulva phenotype characterized by the production of ectopic pseudovulvae (3, 4). Applicants screened for non-Multivulva revertants after ethyl methanesulfonate mutagenesis (5) of *lin-12(n950)* hermaphrodites; two recessive suppressors, *ar131* and *ar133*, proved to be alleles of a new gene, *sel-12* (*sel* means suppressor and/or enhancer of *lin-12*). These *sel-12* alleles cause an incompletely penetrant, recessive egg-laying defective (Egl) phenotype in a *lin-12(+)* background. Since *sel-12(ar131)* is viable, fertile and Egl in trans to a deficiency (data not shown), applicants also performed a screen for mutations that fail to complement the Egl defect of *sel-12(ar131)*. From a screen of 5900 mutagenized haploid genomes, applicants identified two additional *sel-12* alleles. One allele obtained in this screen, *sel-12(ar171)*, displays a completely penetrant Egl defect as a homozygote and in trans to a deficiency, suggesting that *sel-12(ar171)* strongly reduces *sel-12* function. This inference is supported by the molecular analysis described below, which revealed that the *ar171* lesion would result in a truncated protein product.

The Egl phenotype caused by *sel-12* mutations in a *lin-12(+)* background is reminiscent of the Egl phenotype caused by reducing *lin-12* activity (see Table 1 legend). However, a more general involvement of *sel-12* in *lin-12-* and *glp-1*-mediated cell fate decisions becomes apparent when the phenotypes of *lin-12; sel-12* and *glp-1; sel-12* double mutants are analyzed

- (Table 1). Applicants examined the genetic interactions of *sel-12* with two *lin-12* hypomorphic mutations, with a *lin-12(d)* hypermorphic mutation, and with a *glp-1* hypomorphic mutation. In all cases, applicants found that reducing *sel-12* activity reduces *lin-12* or *glp-1* activity. These genetic interactions are exemplified by the effects of *sel-12* on two *lin-12*-mediated decisions, the anchor cell/ventral uterine precursor cell (AC/VU) decision and vulval precursor cell (VPC) specification.
- 10 The AC/VU decision involves an interaction between two initially equivalent cells of the somatic gonad, Z1.ppp and Z4.aaa. In a given hermaphrodite, Z1.ppp and Z4.aaa interact so that one of these cells becomes the AC while the other becomes a VU (6, 7, 8). When *lin-12* activity is eliminated, both Z1.ppp and Z4.aaa become ACs (the "2 AC defect"), and when LIN-12 is activated, as in *lin-12(d)* mutants, both Z1.ppp and Z4.aaa become VUs (the "0 AC defect") (3,9). Two observations indicate that *sel-12* reduces *lin-12* activity in Z1.ppp and Z4.aaa. First, *sel-12* dramatically enhances the penetrance of the 2 AC defect of *lin-12* hypomorphs (Table 1A). For example, 30% of *lin-12(n676n930)* hermaphrodites have 2 AC (10), whereas essentially all *lin-12(n676n930); sel-12(ar171)* have 2 ACs. Second, *sel-12* partially suppresses the 0 AC defect caused by LIN-12 activation (Table 1B). For example, all *lin-12(n950)* hermaphrodites lack an AC, whereas 10% of *lin-12(n950); sel-12(ar171)* hermaphrodites have an AC.

Table 1.

sel-12(ar171) reduces lin-12 and glp-1 activity

5 A. Enhancement of hypomorphic lin-12 alleles by sel-12 (ar171)

	<u>Genotype</u>	<u>% 2ACs</u>	<u>% ventral coelomocytes</u>	<u>fertility</u>	<u>% L1 arrest<sup>k</sup></u>
10	wild type <sup>a</sup>	0	0	yes	0
	sel-12(ar171) <sup>b</sup>	0	0 (0/17)	yes	0 (n=233)
15	lin-12(n676n930) <sup>c</sup>	30g	8 (1/12)	yes	9 (n=233)
	lin-12(n676n930); sel-12(ar171) <sup>d</sup>	95 (n=41)	92 (12/13)	no	17 (n=177)
20	lin-12(ar170) <sup>e</sup>	16 (n=32)	0 (0/32)	yes	0 (n=209) <sup>i</sup>
	lin-12(ar170); sel-12(ar171) <sup>f</sup>	98 (n=47)	0 (0/47)	yes	0 (n=111)
25	lin-12(O)	100 <sup>h</sup>	100 <sup>h</sup>	no	10 <sup>j</sup>

30

35 B. Suppression of a hypermorphic lin-12 allele by sel-12(ar171)

	<u>Genotype</u>	<u>number of VPCs adopting a vulval fate/hermaphrodite</u>	<u>% 0 AC</u>
40	wild type <sup>a</sup>	3	0
	lin-12(n950) <sup>1</sup>	6 (n=7)	100
	sel-12(ar171) <sup>b</sup>	3 (n=10)	0 (n=108)
45	lin-12(n950); sel-12(ar171) <sup>m</sup>	2-4 (n=8)	89.5 (n=57)

50

C. Enhancement of glp-1(e2141) by sel-12(ar171)

	<u>Genotype</u>	<u>% sterility in both gonad arms</u>	<u>% sterility in one gonad arm</u>
5	wild type <sup>a</sup>	0	0
	glp-1(e2141) <sup>n</sup>	8.5 (n=259)	4.0 (n=259)
10	sel-12(ar171) <sup>b</sup>	0	0
	glp-1(e2141); sel-12(ar171) <sup>o</sup>	25 (n=422)	8.8 (n=422)
15	<sup>a</sup> C. elegans var. Bristol strain N2 <sup>b</sup> sel-12(ar171) unc-1(e538) <sup>c</sup> lin-12(n676n930); unc-1(e538) <sup>d</sup> lin-12(n676n930); sel-12(ar171) unc-1(e538) <sup>e</sup> lin-12(ar170); unc-1(e538) <sup>f</sup> lin-12(ar170); sel-12(ar171) unc-1(e538) <sup>g</sup> see ref. 10 <sup>h</sup> lin-12(n137n720); see ref. 3 <sup>i</sup> lin-12(ar170) [not unc-1] <sup>j</sup> lin-12(n941) see ref. 23 <sup>k</sup> some L1 arrested animals were examined for Lag phenotypes, i.e. lack of an anus and rectum, lack of an excretory cell and a twisted nose. These phenotypes were observed for all genotypes where L1 arrested animals were identified. <sup>l</sup> lin-12(n950); unc-1(e538) <sup>m</sup> lin-12(n950); sel-12(ar171) unc-1(e538) <sup>n</sup> glp-1(e2141); unc-1(e538) <sup>o</sup> glp-1(e2141); sel-12(ar171) unc-1(e538)		
20			
25			
30			
35			

Table 1. Legend

Most lin-12- and glp-1-mediated cell fate decisions appear normal in sel-12(ar171) mutants. However, the egg-laying defect of sel-12(ar171) hermaphrodites resembles the egg-laying defect of lin-12 hypomorphic mutants (10): sel-12(ar131) hermaphrodites leak occasional eggs and larvae, and like lin-12 hypomorphic mutants, sel-12 mutants have morphologically normal HSNs, sex muscles and VPC lineages. Egg-laying is particularly sensitive to reduction in lin-12 activity (10); H. Wilkinson and I.G., unpublished observations). It is therefore possible that both lin-12 and sel-12 are required for an as yet unidentified cell fate decision(s) underlying the egg-laying defect. The fact that sel-12(ar171) mutants do not display all of the defects associated with loss of lin-12 function may indicate that sel-12(ar171) is not a null allele or sel-12



function is partially redundant with the function of another gene.

- 5 A. Cell fate transformations were scored at 25° using criteria described in (3) unless otherwise indicated. At 25° *lin-12(n676n930)* behaves like a hypomorph, whereas at 15°C, *lin-12(n676n930)* has mildly elevated *lin-12* activity (10). Since *lin-12(n676n930); sel-12(ar171)* hermaphrodites are sterile at 25°C, applicants shifted 10 fertile *lin-12(n676n930); sel-12(ar171)* hermaphrodites from 15°C to 25°C so that their progeny could be scored for cell fate transformations and other defects. *lin-12(ar170)* behaves like a hypomorph for the AC/VU decision (J. Hubbard and I.G., unpublished observations). In 15 strains containing *lin-12(ar170)*, cell fate transformations were scored in hermaphrodites raised at 20°; other defects were scored in the progeny of hermaphrodites grown at 20° and shifted to 25°.
- 20 % 2ACs : In *lin-12(0)* mutants, both Z1.ppp and Z4.aaa become ACs, so *lin-12(0)* hermaphrodites have two ACs; in *lin-12(d)* mutants such as *lin-12(n950)*, both Z1.ppp and Z4.aaa become VUs, so *lin-12(d)* hermaphrodites have 0 ACs. The number of anchor cells was scored in the L3 stage 25 using Nomarski microscopy. For all genotypes, hermaphrodites either had one or two ACs.
- 30 ventral coelomocytes: The fates of two pairs of cells, M.d(l/r)pa and M.v(l/r)pa are affected by mutations in *lin-12*. In wild type, the ventral pair of cells gives rise to one sex-myoblast and one body muscle; the dorsal pair gives rise to coelomocytes. In *lin-12(0)* animals, the ventral pair as well as the dorsal pair gives rise to coelomocytes, so that *lin-12(0)* hermaphrodites have extra 35 ventral coelomocytes; in *lin-12(d)* animals, both pairs of cells give rise to sex myoblasts/body muscles. The presence of ventral coelomocytes was scored in the L3 stage. For all genotypes, the absence of ventral coelomocytes suggests that the sex myoblast was specified

normally (see ref. 3).

5 Fertility: fertility was scored by the appearance of eggs either on the plate or inside the hermaphrodite and the ability to propagate the strain.

10 L1 arrest: Full viability requires activity of *lin-12* or a related gene, *glp-1*. *lin-12(0) glp-1(0)* double mutants display a fully penetrant L1 arrest phenotype and a Lag phenotype characterized by specific cell fate transformations (23). *lin-12(0)* single mutants display a low penetrance L1 arrest phenotype and a somewhat lower penetrance Lag phenotype (23). Single gravid hermaphrodites were placed on a plate at 25°C. Most of the hermaphrodites were completely egg-laying defective and laid no eggs; some *lin-12(n676n930)* animals released a few eggs or larvae before turning into "bags of worms", in which case the hermaphrodite was transferred after a day. Since *lin-12(n676n930)* animals can grow slowly at 25°C, L1 arrested animals were scored three days after all the eggs had hatched. Arrested L1 animals were spot-checked for the presence of Lag phenotypes using Nomarski microscopy. Some arrested L1 animals of each genotype displayed Lag phenotypes (data not shown).

25 B. Animals were grown at 20°C. VPC fates were scored by determining the cell lineages of P3.p-P8.p in each animal (Table 2 and data not shown). The number of ACs were scored as described above. For all genotypes, hermaphrodites had either zero or one AC.

30 C. *glp-1(e2141ts)* is weakly hypomorphic at 20° and essentially wild-type at 15° (24). Strains containing *glp-1(e2141)* were maintained at 15°; fertile adults grown at 15° were placed at 20°, and their progeny grown at 20° were scored for sterility. Other strains were maintained continuously at 20°. *glp-1* activity controls the decision of germline nuclei between mitosis and meiosis (25, 24); L. W. Berry and T. Schedl, personal communication). GLP-1

is thought to be the receptor for the inductive signal from the distal tip cells of the somatic gonad that promotes germline mitosis (and/or inhibits meiosis) (7). When *glp-1* activity is eliminated, germline nuclei enter meiosis (25). Hermaphrodites of each genotype were scored for sterility in one or both gonad arms in the dissecting microscope. Several sterile or half-sterile individuals were examined by Nomarski microscopy, and sterile gonad arms were found to have the characteristic Glp phenotype (data not shown).

Each of the six VPCs, P3.p-P8.p, has the potential to adopt one of two vulval fates, termed "1°" and "2°", or a non-vulval fate, termed "3°" (11, 12). Normally, P5.p, P6.p, and P7.p adopt vulval fates, in a 2°-1°-2° pattern (13). This pattern is the outcome of the integration of two signalling inputs: a *let-60* Ras-mediated inductive signal from the AC induces vulval fates, and a *lin-12*-mediated lateral signal between VPCs prevents adjacent VPCs from adopting the 1° fate (reviewed in ref. 14). The *let-60* Ras-mediated inductive signal may cause expression or activation of the lateral signal (15, 16), which activates LIN-12 to cause a VPC to adopt the 2° fate (3, 17, 18).

Reducing *sel-12* activity reduces *lin-12* activity in lateral signalling that specifies the 2° fate of VPCs. First, *sel-12* reduces the effect of activated LIN-12 in the VPCs: all VPCs adopt the 2° fate in *lin-12(n950)* hermaphrodites, but only half of the VPCs adopt the 2° fate in *lin-12(n950); sel-12(ar171)* hermaphrodites (Table 1b, Table 2). Second, *sel-12* reduces lateral signalling that occurs upon activation of *let-60* Ras. Applicants analyzed VPC lineages (data not shown) in *let-60(n1046)* hermaphrodites, in which Ras has been activated by a codon 13 mutation (19, 20), and in *let-60(n1046); sel-12(ar171)* hermaphrodites. Lateral signalling appears to occur normally in *let-60(n1046)* hermaphrodites, since adjacent VPCs do not adopt the 1° fate (0/20 pairs of induced VPCs). In contrast, adjacent VPCs sometimes adopt the 1° fate in *let-60(n1046);*

*sel-12(ar171)* hermaphrodites (4/18 pairs), implying that reducing the activity of *sel-12* reduces lateral signalling. Finally, some VPCs adopt the 2° fate in *lin-12(n676n930)* hermaphrodites (10). In contrast, VPCs do not adopt the 2° fate in *lin-12(n676n930); sel-12(ar171)* double mutants (data not shown), although applicants have not tested whether this effect is due to the presence of a second AC.

Table 2.

*sel-12(ar171)* plays a role in the receiving cells

Genotype	Expression of 2° fate/total						% VPCs adopting a 2° fate hermaphrodite
	P3.p	P4.p	P5.p	P6.p	P7.p	P8.p	
<i>lin-12(n950)</i>	7/7	7/7	7/7	7/7	7/7	7/7	100
<i>lin-12(n950); sel-12(ar171)</i>	0/8	1/8	4/8*	8/8	6/8	2/8**	52
<i>lin-12(n-950)</i>	X	11/11	X	X	X	X	100
<i>lin-12(n950); sel-12(ar171)</i>	X	3/10	X	X	X	X	30

### Table 2. Legend

X=cell killed by a laser microbeam. Numbers in each column correspond to the proportion of times a given VPC was observed to adopt the 2° fate (criteria as in ref. 18). All VPCs that did not undergo 2° fates underwent 3°, or non-vulval fates, with three exceptions: \* = in 1/8 animals examined, P5.p underwent a hybrid (2°/3°) lineage; \*\* = in 2/8 animals examined, P8.p underwent a hybrid (2°/3°) lineage. Animals were maintained at 20°C. Early L2 hermaphrodites (as judged by the size of the gonad) were chosen for laser ablation studies. The fates of the VPCs have not been determined at this time; the VPCs become determined many hours later, in the L3 stage (Sternberg and Horvitz, 1986). P3.p, and P5.p-P8.p were destroyed with a laser microbeam; the success of this operation was verified 2-3 hours later. The following day, the operated animals were mounted for Nomarski microscopy so that the cell lineage of P4.p could be observed directly. In both operated

and unoperated animals, vulval fates were scored by directly observing the cell lineage of each VPC. The operated animals were observed until the early L4 stage, to ensure that no divisions were missed.

5

The genetic interactions of *sel-12* with *lin-12* imply a function for *sel-12* in signalling and/or receiving cells during lateral  
10 specification. Applicants have tested whether *sel-12* functions in the receiving end of *lin-12*-mediated cell-cell interactions by performing cell ablation experiments (Table 2). Applicants reasoned that, if all VPCs but one were ablated with a laser  
15 microbeam, the fate of the isolated VPC would reflect its intrinsic level of *lin-12* activity in the absence of lateral signal. Thus, in *lin-12(n950)* hermaphrodites, an isolated VPC adopts the 2° fate (Table 2), suggesting that it has a high level of ligand-independent activation of LIN-12 in the VPCs  
20 (9). If *sel-12* were to function in one VPC to lower *lin-12* activity in another, then in *lin-12(n950); sel-12(ar171)* hermaphrodites, an isolated VPC should also adopt the 2° fate. However, if *sel-12* were to function within a VPC to lower its  
25 *lin-12* activity, then in *lin-12(n950); sel-12(ar171)* hermaphrodites, an isolated VPC should instead adopt the 3° fate. Applicants observed that in *lin-12(n950); sel-12(ar171)* hermaphrodites, an isolated P4.p often adopts the 3° fate (Table 2), implying that *sel-12* functions within a VPC to lower  
30 *lin-12* activity.

30 Applicants cloned *sel-12* by transformation rescue (Fig. 1 legend), and determined the nucleotide sequence of a full-length cDNA (Genbank Accession number U35660). The predicted  
35 SEL-12 protein contains multiple potential transmembrane domains (Fig. 1B), consistent with SEL-12 function as a receptor, ligand, channel, or membrane structural protein. The SEL-12 protein is evolutionarily conserved. Database searches revealed a high degree of similarity to a sequence of a partial  
cDNA from human brain present on clone T03796 and a low degree of similarity to SPE-4, a protein required for *C. elegans*

- spermatogenesis (21). In addition, SEL-12 is highly similar to S182, which, when mutant, has been implicated in familial early-onset Alzheimer's Disease (22). T03796 has recently been shown to correspond to the E5-1/STM2 gene, which has also been
- 5 implicated in early onset familial Alzheimer's disease (Levy-Lahad et al., 1995a,b; Rogaev et al., 1995). The predicted protein sequences of SEL-12, ES-1/STM2, SPE-4, and S182 are aligned in Fig. 2.
- 10 *lin-12/Notch* genes specify many different cell fate decisions in *C. elegans* and *Drosophila*, and in both organisms some of these decisions are critical for neurogenesis. The genetic analysis described here indicates that *sel-12* facilitates *lin-12*-mediated reception of intercellular signals. *sel-12* might
- 15 be directly involved in *lin-12*-mediated reception, functioning for example as a co-receptor or as a downstream effector that is activated upon LIN-12 activation. Alternatively, *sel-12* may be involved in a more general cellular process such as receptor localization or recycling and hence influence *lin-12* activity
- 20 indirectly. Although the remarkable conservation of *sel-12* and S182 does not provide any immediate indication of the function of S182 in the Alzheimer's disease process, it is striking that 4 of the 5 mutations found in affected individuals alter amino acids that are identical in SEL-12 and
- 25 S182 (see Fig. 2). The powerful tools of classical and molecular genetic studies in *C. elegans*, including the ability to identify extragenic suppressor and to generate transgenic lines containing engineered genes, can now be brought to bear on fundamental issues of SEL-12/S182 structure and function.

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## Second Series of Experiments

### BACKGROUND AND SIGNIFICANCE

Alzheimer's disease is a devastating and common disease of the  
5 central nervous system, and studies of familial forms have  
identified a number of loci that are implicated in the  
development of the disease. Two loci, S182 (AD3) (Sherrington  
et al., 1995) and STM2 (Levy-Lahad et al., 1995a,b), which is  
also known as E5-1 (Rogaev et al., 1995), have recently been  
10 found to be associated with the development of early onset  
familial Alzheimer's disease. These loci encode related  
proteins with multiple transmembrane domains.

The *C. elegans* model described here is based on the finding  
15 that the *sel-12* gene encodes a protein that is highly similar  
to S182 and STM2 (Levitan and Greenwald, 1995; see Fig. 1).  
For example, SEL-12 and S182 are 48% identical over 460 amino  
acids. The remarkable conservation of the SEL-12 and S182  
predicted protein structure suggests that their functions are  
20 likely to be conserved as well. Furthermore, it is striking  
that seven of the eight changes in S182 that are associated  
with early-onset familial Alzheimer's disease (Rogaev et al.,  
1995; Sherrington et al., 1995; see Fig. 1) alter amino acids  
that are identical in SEL-12, and that the eighth alters an  
25 amino acid that has been changed very conservatively during  
evolution, and two out of two changes in STM2/E5-1 that are  
associated with Alzheimer's disease (Levy-Lahad et al., 1995b;  
Rogaev et al., 1995) affect amino acids that are identical in  
SEL-12.

30 Applicants hope to bring the powerful tools of classical and  
molecular genetic studies in *C. elegans* to bear on fundamental  
issues of SEL-12/S182/STM2 structure and function. Thus far,  
proteins similar to LIN-12/Notch and SEL-12/S182/STM2 have not  
35 been described in single-celled organisms (for example, >95% of  
the yeast genome has been sequenced and has not yielded any  
similar proteins), so *C. elegans* may be the simplest practical  
system for studying these issues in vivo.

## PRELIMINARY STUDIES

*sel-12*. Applicants identified *sel-12* [*sel* = suppressor/enhancer of *lin-12*] by screening for suppressors of the "Multivulva" phenotype caused by an allele of *lin-12* that causes  
5 constitutive LIN-12 activation. Applicants performed a genetic and molecular characterization of *sel-12* (Levitan and Greenwald, 1995), which established: (1) Reducing or eliminating *sel-12* activity reduces the activity of *lin-12* and of *glp-1*, another member of the *lin-12*/Notch family. In  
10 addition, reducing or eliminating *sel-12* activity causes an egg-laying defective (Egl) phenotype. Applicants do not know if the Egl phenotype is a direct consequence of reducing *lin-12* activity (Sundaram and Greenwald, 1993a) or an independent effect of reducing *sel-12* activity. (2) *sel-12* and *lin-12* can  
15 functionally interact within the same cell. (3) *sel-12* is predicted to encode a protein with multiple transmembrane domains that is highly similar to S182 and STM2, which have been implicated in early-onset familial Alzheimer's disease (Levy-Lahad et al., 1995a, b; Rogaev et al., 1995; Sherrington  
20 et al., 1995). The presence of multiple transmembrane domains is consistent with SEL-12 function as a receptor, ligand, channel or membrane structural protein.

The fact that the only striking phenotype caused by *sel-12(ar171)* is a defect in egg-laying may reflect the fact that  
25 egg-laying is particularly sensitive to reduction in *lin-12* activity (Sundaram and Greenwald, 1993a; H. Wilkinson and I.G., unpublished observations). The egg-laying defect may reflect an as yet unidentified cell fate decision(s), or alternatively  
30 may also be viewed as a late-onset behavioral phenotype. However, the fact that *sel-12(ar171)* mutants do not display all of the defects associated with loss of *lin-12* function may indicate that *sel-12(ar171)* is not a null allele, despite the severe truncation in protein product it is expected to cause;  
35 alternatively, *sel-12* function may be partially redundant with the function of another gene.

Applicants identified a genomic fragment capable of complementing *sel-12* alleles (Levitan and Greenwald, 1995).

Some of the experiments described in this invention require the ability to express reporter genes or altered *sel-12* genes appropriately. An expression method developed in applicants' laboratory will enable these experiments to be performed. (1) Applicants have developed a vector that expresses inserted cDNAs under the control of *lin-12* regulatory sequences (pLEX; Struhl et al., 1993). The applicants have found that construct containing a *sel-12* cDNA in the pLEX vector is capable of rescuing *sel-12* mutants. (2) Applicants have developed an analogous vector, p1B7, that should express inserted cDNAs under the control of *sel-12* regulatory sequences. p1B7 is based on a genomic fragment that is capable of rescuing *sel-12* mutants (Levitan and Greenwald, 1995): a unique BamHI site was inserted at +1 into a genomic fragment capable of complementing a mutant allele, thereby destroying the first codon of the gene. The expression vector contains 3.5 kb of 5' flanking region (2.5 kb more than the original rescuing fragment of Levitan and Greenwald, 1995) and 0.5 kb of 3' flanking region.

These vectors are used as follows (Wilkinson et al., 1994; Fitzgerald and Greenwald, 1995; Wilkinson and Greenwald, 1995). A cDNA containing its own start and stop codons, but lacking a polyadenylation signal, is inserted into the vector. The resulting transcript is predicted to contain an unusually long 3' untranslated region (UTR). These aberrant 3' UTRs are generally destabilizing, leading to very low levels of detectable expression. However, this problem can be overcome by placing the transgenes in a *smg* mutant background, which stabilizes mRNAs with long 3' untranslated regions (Pulak and Anderson, 1993). The recent identification of a temperature-sensitive *smg-7* mutation (B. Cali and P. Anderson, personal communication) enables transgenic lines to be generated at the permissive temperature (15°), where *smg-7(ts)* has nearly wild-type activity, and shifted to the restrictive temperature (25°) for the analysis of mutant phenotypes (K. Fitzgerald, personal communication).

*lin-12*. *lin-12* is the archetype of the "*lin-12/Notch* gene family" of putative transmembrane receptor proteins that is

found throughout the animal kingdom (reviewed in Greenwald and Rubin, 1992; Artavanis-Tsakonas et al., 1995). Members of this family are transmembrane proteins with repeated epidermal growth factor (EGF)-like motifs and LIN-12/Notch repeat motifs in their extracellular domains, and "cdcl0/SWI6" motifs (also termed "ankyrin repeats") in the intracellular domains. In *C. elegans* and *Drosophila*, *lin-12/Notch* family members were first defined genetically, by mutations that alter cell fate decisions that involve cell-cell interactions during development (reviewed in Greenwald and Rubin, 1992). In vertebrates, *lin-12/Notch* genes were identified either by cross-hybridization with *Notch* probes, or, more revealingly, by oncogenic mutations: mutation of *int-3* by mouse mammary tumor virus is associated with the development of breast cancer in mice (Gallahan and Callahan, 1987; Robbins et al., 1992) and mutation of *TAN-1* is associated with T cell leukemias in people (Ellisen et al., 1991; Robbins et al., 1992).

The nature of the relationship between *lin-12* and *sel-12* is uncertain. *lin-12/Notch* genes specify many different cell fate decisions in *C. elegans* and *Drosophila*, and in both organisms some of these decisions are critical for neurogenesis. As described above, the initial genetic analysis indicated that *sel-12* facilitates *lin-12*-mediated reception of intercellular signals (Levitan and Greenwald, 1995). *sel-12* might be directly involved in *lin-12*-mediated reception, functioning for example as a co-receptor or as a downstream effector that is activated upon LIN-12 activation. Alternatively, *sel-12* may be involved in a more general cellular process such as receptor localization or recycling and hence influence *lin-12* activity indirectly. The powerful tools of classical and molecular genetic studies in *C. elegans*, including the ability to identify extragenic suppressors and to generate transgenic lines containing engineered genes, can now be brought to bear on fundamental issues of SEL-12/S182/STM2 structure and function.

## RESEARCH DESIGN AND METHODS

### I. Basic characterization of *sel-12*.

A. Additional basic characterization of *sel-12*. There are several lines of experimentation that, along with previous work (Levitan and Greenwald, 1995), will constitute the basic characterization of *sel-12*.

5

(1) Null phenotype. Although *sel-12(ar171)* is predicted to encode a protein that is truncated by half, it is conceivable that this portion of the protein retains some activity and that *sel-12(ar171)* is not a true null allele [*sel-12(ar171)* mutants have normal mRNA levels]. Null alleles will be used to reveal the requirement for gene activity, for gene dosage studies, and as a background into which engineered *sel-12* mutations can be introduced. Applicants will therefore isolate additional *sel-12* alleles by complementation screening as described in Levitan and Greenwald (1995), with the goal of identifying an internal deletion of *sel-12* or an allele associated with a stop codon early in the gene. If alleles with early stops or internal deletions cause a more severe phenotype than *sel-12(ar171)*, applicants will analyze the phenotype in detail. Alleles with other properties may also be obtained from the screen + may be useful for other experiments, such as drug testing.

(2) Expression pattern. Using the expression vector p1B7 applicants have engineered a *sel-12::lacZ* reporter gene. The *lacZ* gene used contains a nuclear localization signal (Fire et al., 1990), which facilitates the identification of individual cells. A developmental profile of expression will be determined. Preliminary results indicate that *sel-12::lacZ* is more broadly expressed than *lin-12::lacZ* (Wilkinson and Greenwald, 1995), including much expression in the nervous system.

(3) Behavioral defects. Besides the Egl defect of hermaphrodites, there may be other behavioral defects. For example, preliminary results suggest that *sel-12(ar171)* males display behavioral abnormalities that affect mating efficiency. Applicants will examine this potential defect further using mating assays (Hodgkin, 1983; Liu and Sternberg, 1994). The *sel-12::lacZ* expression pattern may provide clues for behaviors

that may be affected in *sel-12* mutants.

- (4) **SEL-12 antibodies.** Applicants will use standard methods (Harlow and Lane, 1988) to generate antibodies to SEL-12. The antibodies will be useful for examining protein localization: the localization of wild-type and mutant SEL-12 proteins in otherwise wild-type backgrounds and in suppressor mutant backgrounds.
- (5) **Identification of *C. elegans* genes that are highly related to SEL-12.** One possible reason that the phenotype of *sel-12(ar171)* is of relatively limited severity is that *sel-12* is partially functionally redundant with another gene or genes. Functional redundancy might be reflected in sequence similarity. The *C. elegans spe-4* gene (L'Hernault and Arduengo, 1992) is weakly related to *sel-12* (see Fig.1) and in collaboration with Steve L'Hernault (Emory University), applicants will express a *spe-4* cDNA under the control of *sel-12* or *lin-12* regulatory sequences, to see if SPE-4 can replace SEL-12. Applicants will also examine the phenotype of *spe-4*; *sel-12* double mutants to see if the double mutant has a more severe phenotype than either single mutant.

If more closely related genes exist, applicants can easily identify them by periodically searching the database of the *C. elegans* sequencing project, which is currently 25% complete, and is expected to be fully completed by 1998 (R. Waterston et al., personal communication). It may also be possible to identify *sel-12* related genes by low-stringency hybridization (Sambrook et al., 1989) and/or screening an expression library with SEL-12 antibodies (Harlow and Lane, 1988). If any method identifies genes that are related to *sel-12*, applicants will express them under the control of *sel-12* or *lin-12* regulatory sequences to see if they can functionally replace *sel-12*. If so, then applicant will attempt to generate null alleles of the *sel-12*-related gene, using a Tc1 transposon-based excision method (Rushforth et al., 1993; Zwaal et al., 1993; Greenwald et al., 1994), unless better gene "knock-out" technology becomes available. The phenotype of null mutants will be

examined alone, and in combination with *sel-12*(null).

It is also possible that genes similar to *sel-12* will be revealed by the analysis of other genes identified by reverting  
5 alleles of *lin-12* (Sundaram and Greenwald, 1993b; J. Thomas, F. Tax, E. Ferguson and H.R. Horvitz, personal communication; D. Levitan and I. Greenwald., unpublished observations).

**B. Functional equivalence of S182, STM2 and SEL-12.** There is  
10 high degree of similarity between SEL-12, S182, and STM2, which suggests they have similar biochemical functions and properties. The best test of this hypothesis would be to demonstrate that S182 and STM2 can substitute for SEL-12. Applicants will place the human cDNAs under the control of *sel-*  
15 *12* regulatory sequences, using the p1B7 expression vector and will assess the ability of S182 or STM2 to replace SEL-12 in *C. elegans*.

**II. Engineered *sel-12* transgenes ["*sel-12*(Alz)"] containing**  
20 **alterations associated with early-onset familial Alzheimer's disease**

The experiments in this section of the proposal are designed to help understand the consequences of mutation of S182 and STM2  
25 for protein function. Mutations that alter the SEL-12 protein so that they resemble mutant proteins associated with familial early-onset Alzheimer's disease will be created. Because genetic analysis in *C. elegans* has revealed the phenotypic consequences of reducing *sel-12* activity as well as the  
30 phenotypic consequences of both reduced and elevated *lin-12* activity, genetic analysis of phenotypes associated with *sel-12*(Alz) mutations will reveal the effect of S182 and STM2 mutations on S182 and STM2 function.

35 **A. Generation of transgenic *C. elegans* lines.** Applicants will create engineered *sel-12* transgenes containing alterations associated with early-onset familial Alzheimer's disease in people. Applicants will engineer the changes using standard PCR-based strategies in a clone of *sel-12* genomic DNA. These

clones will be microinjected into *lin-12(+)*; *sel-12(+)* *C. elegans* (either the wild-type strain N2 or usefully marked derivatives) to establish transgenic lines (Fire, 1986; Mello et al., 1991), which will be analyzed for mutant phenotypes and for interactions with *lin-12*. The *rol-6(sul1004)* gene (Mello et al., 1991) will be used as a cotransformation marker; other cloned genes may be used as cotransformation markers to facilitate phenotypic analysis, which can be difficult in Roller mutants, if necessary. Several different concentrations of injected DNA will be tried.

Table 3.

	Human gene	Mutation	SEL-12 residue
15	S182	M146L	M115
		H163R	H132
		A246E	V215
		A260V	A229
		A285V	A254
20		L286V	L255
		L382V	L371
		C410Y	C387
25	STM2	N141I	N104
		M239V	M202

Table 3. Mutations associated with the development of Alzheimer's disease (Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995), and the corresponding amino acid in SEL-12 (see also Figure 1). Note that nine of ten mutations in S182 or STM2 affect amino acids that are identical in SEL-12. The tenth, S182 A246E, causes a dramatic change in a residue that is conservatively different between S182 and SEL-12.

If the *sel-12(Alz)* mutations cause dominant lethal or sterile phenotypes that prevent the establishment of transgenic lines, applicants will use an alternative strategy to achieve conditional or more limited expression. The engineered mutations will be incorporated into a *sel-12* cDNA, which can be cloned into a *sel-12* expression vector applicants have made (see "Background and Preliminary Studies"): in this vector, the ATG of the cloned *sel-12(+)* gene has been replaced by a BamHI linker, so that cDNAs can be cloned into the unique BamHI site and expressed under the control of *sel-12* regulatory sequences. Efficient expression should be obtained in a *smg*



mutant background, so that transgenic arrays may be generated in a *smg(+)* background and crossed into a *smg* background for analysis, or generated in a *smg-7(ts)* background at the permissive temperature (15°) and analyzed at the restrictive  
5 temperature (25°). The temperature-sensitive *smg-7* mutant will be particularly useful, since transgenic worms may be shifted at different times during development, and the effects on different cell fate decisions examined.

- 10 Applicants can also clone the mutant *sel-12* cDNAs into a *lin-12* expression vector (Struhl et al., 1993), which has a more restricted pattern of expression (defined by Wilkinson et al., 1994; Wilkinson and Greenwald, 1995) and hence may be less deleterious. Although heat shock promoter-based vectors are  
15 available, in applicants' experience they have not been reliably effective for studies of *lin-12*-mediated cell fate decisions, probably because of tissue-specificity of the heat shock promoters (see Stringham, Fire). However, they may be useful for examining the consequences of altered *sel-12* coding  
20 regions in other tissues, or for ectopic expression experiments.

Applicants can also perform analogous experiments using mutated human *S182* or *E5-1/STM2* cDNAs cloned into p1B7 or pLEX.

- 25 Applicants will create integrated lines for phenotypic analysis. In *C. elegans*, the microinjection technique used to establish transgenic lines generally results in lines containing extrachromosomal arrays of injected DNAs. Such  
30 extrachromosomal arrays may be integrated by irradiation (Hedgecock and Herman, 1995), so that arrays become inserted randomly into the genome. Such lines generally have more reproducible expression from the transgenes, and avoid complications for phenotypic analysis introduced by the  
35 potential for somatic mosaicism of extrachromosomal arrays.

**B. Phenotypic analysis of transgenic lines containing *sel-12(Alz)* genes.** Integrated lines carrying *sel-12(Alz)* genes will be analyzed for viability and fertility. They will also

be examined for the Egl phenotype associated with reduced *sel-12* activity (Levitan and Greenwald, 1995), and other phenotypes that may be revealed by the analysis described in section I of this proposal. They will also be analyzed for phenotypes associated with reduced *lin-12* activity (such as 2 anchor cells, no 2° vulval precursor cell lineages, ventral coelomocytes/missing sex muscles; Greenwald et al., 1983; Sundaram and Greenwald, 1993a) or elevated *lin-12* activity (such as no anchor cell, ectopic 2° vulval precursor cell lineages, extra sex muscles/no dorsal coelomocytes; Greenwald et al., 1983), and reduced *glp-1* activity (such as germline proliferation defect, missing anterior pharynx or extra pharyngeal cells; Austin and Kimble, 1987; Priess et al., 1987; Bowerman et al., 1994; Mello et al., 1994) or elevated *glp-1* activity (Fitzgerald and Greenwald, 1995; tumorous germ line; L. W. Berry and T. Schedl, personal communication).

If it is necessary to use a conditional expression system to generate the lines, transgenic animals will be examined after a shift from the permissive to the restrictive temperature at different times during development.

If antibodies to SEL-12 are available, the localization of wild-type and mutant SEL-12 proteins will be examined by examining stained whole-mounts by confocal microscopy and possibly by immunoelectron microscopy.

**C. Genetic analysis of *sel-12(Alz)* genes.** The S182 and STM2 mutations associated with early onset Alzheimer's disease in people are dominant. The most likely possibility is that altered gene activity underlies this dominance, since ten different mutations in S182 and STM2 are missense mutations in conserved amino acids. Dominant mutations may cause a mutant protein to have elevated activity, decreased activity, or aberrant activity. Genetic tests can be used to distinguish these possibilities, and are particularly valuable when biochemical function is not known or when biochemical assays are difficult to execute on mutant proteins. Thus, the ability to assess the genetic properties of the *sel-12(Alz)* transgenes

in *C. elegans*, where rigorous genetic tests to determine the consequences of mutation on gene activity are possible, may be very valuable for understanding the effect of the mutations on Alzheimer's disease loci in people.

5

If *sel-12(Alz)* mutations cause dominant phenotypes in *C. elegans* (i.e., phenotypes in a *sel-12(+)* background), applicants will examine them by adapting classical gene-dosage tests (Muller, 1932) for hypermorphic (elevated), neomorphic  
10 (novel) or antimorphic (dominant-negative) activity. Two approaches will be used. First, established arrays carrying *sel-12(Alz)* genes will be crossed into *sel-12(ar171)* mutants, and into *sel-12(+)* hermaphrodites carrying a duplication of *sel-12(+)*. Second, additional arrays will be established by  
15 coinjection of *sel-12(Alz)* with *sel-12(+)* genes. If a *sel-12(Alz)* mutation is a hypermorph, then the severity of the mutant phenotype should increase as additional doses of *sel-12(+)* are added. If a *sel-12(Alz)* mutation is a neomorph, then the severity of the mutant phenotype should be essentially  
20 unchanged as additional doses of *sel-12(+)* are added. If a *sel-12(Alz)* mutation is an antimorph, then the severity of the mutant phenotype should decrease as additional doses of *sel-12(+)* are added.

25 If *sel-12(Alz)* does not cause a phenotype in a *sel-12(+)* background, the *sel-12* activity of the transgenes will be assessed by placing the transgenes into a *sel-12(ar171)* or *sel-12(null)* background. If the *sel-12(Alz)* transgenes do not have rescuing activity, then applicants will not be able to draw any  
30 rigorous conclusions.

### III. Identification and characterization of extragenic suppressors of *sel-12(ar171)* and *sel-12(Alz)*

35 Extragenic suppressor mutations may identify new genes that are involved in *SEL-12/S182/STM2*-mediated processes. Even if suppressor mutations identify genes that were defined previously, they will reveal a functional connection with *sel-12/S182/STM2*. Genetic and molecular characterization of these

"suppressor genes" in *C. elegans* will reveal the nature of their interactions with *sel-12* and *lin-12*. Furthermore, if suppressor mutations, or other alleles of suppressor genes that can be subsequently generated (such as null alleles), have  
5 highly-penetrant, easily scored phenotypes, they too can be reverted to identify additional genes that may be involved in *sel-12* function. In this way, a network of interacting genes can be identified, and the normal function, as well as the aberrant function in mutants, can be elucidated.

10

A potential outcome of the suppressor analysis is an insight into the biochemistry of SEL-12/S182/STM2-mediated processes. The best outcome will be if one of the suppressor genes has a known biochemical activity (based on sequence analysis). This  
15 information will be combined with the results of genetic analysis suggesting the nature of the interaction of the suppressor mutations with *sel-12*, and will potentially be useful for the design and testing of therapeutic agents in both *C. elegans* and mammalian models, and ultimately for people. A  
20 second important reason is that human homologs of the suppressor genes themselves may be useful diagnostic reagents. For example, such cloned genes might be used to analyze human pedigrees to reveal the underlying defects in other inherited forms of Alzheimer's disease (and will possibly have some use  
25 for sporadic forms as well).

A. Reversion of *sel-12(ar171)*. *sel-12(ar171)* causes a highly penetrant Egl phenotype. Applicants will generate Egl<sup>+</sup> revertants by mutagenizing *sel-12(ar171)* hermaphrodites with  
30 ethyl methanesulfonate (EMS) (Brenner, 1974) and screening for Egl<sup>+</sup> (normal egg-laying) revertants in the F<sub>1</sub>, F<sub>2</sub>, and F<sub>3</sub> generations. This procedure will enable the identification of dominant, recessive and maternal effect suppressor mutations.

35 Applicants performed a pilot mutagenesis, which indicated that this procedure will yield suppressor mutations: applicants identified two suppressor mutations, including a dominant suppressor that maps near *dpy-10 II* (D. Brousseau, personal communication), in a region of the genome that has been well

characterized genetically (e.g., Sigurdson et al., 1984) and sequenced (R. Waterston et al., personal communication). The suppressor mutations appeared to arise at low frequency, suggesting that they may be specific alterations and not null alleles, but applicants did not perform careful quantitation in their pilot experiment. Future mutageneses for suppressor mutations will be performed quantitatively (see e.g., Greenwald and Horvitz, 1980).

10 B. Reversion of *sel-12(A1z)* mutants. If *sel-12(A1z)* mutations cause a highly penetrant phenotype (such as lethality, sterility, or egg-laying defect), applicants will mutagenize integrated lines and look for revertants.

15 C. Analysis of suppressor ("*sup*") mutations.

(1) Basic genetic analysis. This analysis will include:

(a) Mapping and complementation tests. Applicants will determine if the *sup* mutation is recessive or dominant, precisely map the suppressor mutations and perform complementation testing with candidate genes in the region, and perform inter se complementation testing among recessive *sup* mutations mapping in the same region.

25

(b) Phenotypic analysis. The phenotype of *sup* mutations in a *sel-12(+)* background, and in combination with *lin-12* activated (Greenwald et al., 1983; Greenwald and Seydoux, 1990; Struhl et al., 1993), *lin-12* hypomorphic (Sundaram and Greenwald, 1993a), and *lin-12(null)* (Greenwald et al., 1983) alleles will be examined. The localization of wild-type and mutant SEL-12 proteins will be examined by examining stained whole-mounts by confocal microscopy and possibly by immunoelectron microscopy.

35 (c) Gene dosage studies. Genetic studies will be used to illuminate the effect of the *sup* mutation on *sup* gene activity. For a recessive suppressor, the relative suppression of *sup/Df* and *sup/sup* will be compared; these genotypes will also be examined for additional phenotypes. The genotype *sup/sup/+*

will also be examined if an appropriate duplication is available, since it is possible that the *sup* mutations are recessive gain-of-function and require two copies to suppress *sel-12* mutations.

5

For a dominant suppressor, the relative suppression of *sup/Df*, *sup/+* and *sup/+/+* will be compared, by examining the ability to suppress *sel-12* mutations and by analyzing any associated mutant phenotypes. The rationale is the same as described above: if a *sup* mutation is a hypermorph, then the suppression ability (and/or an associated phenotype) should increase as additional doses of *sup*-(+) are added; if *sup* is a neomorph, then the suppression ability (and/or phenotype) should be essentially unchanged as additional doses of *sup*-(+) are added; and if a *sup* mutation is an antimorph, then the suppression ability (and/or mutant phenotype) should decrease as additional doses of *sel-12*(+) are added.

(d) Null phenotype of *sup* genes. If *sup* mutations are not null alleles, then applicants will perform screens for null mutations. For example, if the *sup* mutations are recessive partial loss-of-function mutations and are viable and fertile in trans to a deficiency, then applicants can screen for *sup*\*/; *sel-12* hermaphrodites that are suppressed (where \* = mutagenized chromosome) (see e.g. Greenwald and Horvitz, 1980). If the *sup* mutations are dominant, then applicants can screen for loss of dominant suppressor activity in *sup* \*/+; *sel-12* hermaphrodites (see e.g. Greenwald and Horvitz, 1982). The null phenotype of *sup* loci may reveal the normal role of *sup* genes.

(2) Molecular analysis. The first phase of molecular analysis involves the molecular cloning and DNA sequence analysis of suppressor genes. Transposon tagging (Greenwald, 1985; Moerman et al., 1986), or transformation screening of clones from the well-correlated genetic and physical maps (Coulson et al., 1988 and personal communication) can be used to clone genes in *C. elegans*. The details of such strategies require the completion of the genetic analysis of the suppressor

mutations. A general overview of such strategies is given below.

Transposon-tagging: Suppressor genes may be cloned by  
5 screening for transposon-associated alleles, using the same  
strategies as can be used for identifying null alleles  
described above. Potential transposon-associated alleles can  
be screened by Southern blotting, using transposon probes  
(e.g., Greenwald, 1985; Moerman et al., 1986), or cosmids in  
10 the region provided by the genome project.

Transformation screening: Suppressor genes defined by loss-of-  
function or antimorphic (dominant-negative) mutations may be  
cloned by transformation "antisuppression": cloned cosmids  
15 provided by the genome project may be used to establish  
transgenic arrays that complement *sup* mutations, thereby  
reversing their ability to suppress mutations in *sel-12*. This  
strategy may also be adapted to clone suppressor genes defined  
by gain-of-function hypermorphic or neomorphic mutations.  
20 After a *sup* mutation has been mapped to a small region of the  
physical map, cosmids from the region can be used to probe a  
Southern blot of DNA made from the *sup* mutant, in the hopes of  
identifying an altered restriction fragment associated with the  
*sup* mutation. If an alteration is not detected, then a  
25 modified transformation screening approach may be used. A  
library can be made from a *sup* mutant, and DNA from the region  
can be identified by probing with mapped cDNAs from the region  
provided by the genome project. The potential *sup* containing  
cosmids can be verified by restriction mapping or DNA  
30 fingerprinting (Coulson et al., 1986), and used for  
transformation experiments based on their dominant suppressor  
activity.

Identification of other genes whose activities are influenced  
35 by *sel-12*. Applicants are testing the genetic interaction of  
*sel-12* alleles with mutations in other secreted or  
transmembrane proteins by constructing and analyzing double  
mutants. This information may reveal other pathways that  
involve *sel-12* activity, and may suggest other human diseases

for which sel-12 is relevant.

Identification of other genes involved in sel-12-mediated processes by the yeast two-hybrid system. Applicants will  
5 apply the yeast two-hybrid system to screen a cDNA library for potential interacting proteins and to screen directly for interaction with LIN-12 and GLP-1. The two-hybrid screen, originally developed by Fields and Song (1989), is a powerful strategy for identifying potential interacting proteins. the  
10 screen relies on the ability of GAL4 to activate transcription of a reporter gene containing GAL4 upstream activation sequences. GAL4 has a DNA binding domain (GBD) and an activation domain (GAD). Normally, the two domains are present in the same polypeptide; if they are separated, GAL4 activity  
15 is abolished. However, if the separated domains are joined to protein sequences that interact with each other, the two domains are brought together, and GAL4 activity is restored. Thus, a yeast strain containing a "bait" fused to the GBD is transformed with a library containing potential GAD fusions,  
20 and a selection or screen for reconstituted GAL-4 activity is used to identify candidates.

The virtue of conducting such a screen in *C. elegans* is the potential for genetic analysis of candidate genes, since in the  
25 absence of a functional analysis it is possible that physical interactions revealed by the two-hybrid method are not meaningful in vivo. Mutations that reduce or eliminate the activity of the candidate gene will be analyzed in *C. elegans*. If the candidate clone maps to a genetically well-characterized  
30 region, applicants will try transformation rescue of the extant mutations. Alternatively, null alleles will be identified using PCR-based screens (Rushforth et al., 1993; Zwaal et al., 1993; Greenstein et al., 1994). The consequences of elevating candidate gene activity will be examined by creating high copy  
35 number transgenic lines or by overexpressing the candidate gene in wild-type and mutant backgrounds. Any candidate genes that appear to be involved in SEL-12-mediated processes by genetic analysis can be used in the same way the suppressor "sup" genes described above could be used.



Th use of *sel-12* mutants for screening for compounds that may ameliorate Alzheimer's disease, and possibly other diseases caused by affecting the activity of members of the *SEL-12/S182/STM2* family. *sel-12* mutants generated by standard genetic and transgenic methods may be use for drug testing. This approach is potentially beneficial for two reasons. First using *C. elegans*, the applicants can analyze the effect of drugs on *sel-12* activity even though the biochemical function of *sel-12* is not known, based on the suppression or enhancement of *sel-12* mutant phenotypes (i.e, egg-laying defect and other phenotypes that will be identified, or the effects of altering *sel-12* activity on *lin-12* activity). For example, the proportion of egg-laying competent *sel-12(ar131)* or *sel-12(ar171)* mutant hermaphrodites may be compared when the mutant worms are cultured in the presence of candidate compounds; an increase in the proportion of egg-laying competent worms in the presence of compound would indicate that *sel-12* activity is increased or bypassed. *sel-12* mutants may also be transiently treated with candidate compounds. If the *sel-12(Alz)* mutations have additional or different phenotypic consequences, transgenic lines containing *sel-12(Alz)* transgenes may also be used to screen for the effect of compound on *sel-12(Alz)* activity. Second, *C. elegans* is easy and inexpensive to cultivate. Thus, a preliminary screening of the effect of compounds on *sel-12* mutants may help to set priorities for drug testing in mammalian system, thereby reducing the expense and shortening the amount of time it takes to identify potential therapeutic agents.

Since *sel-12* mutations affect *lin-12* activity, and mammalian homologues of *lin-12* have been implicated in oncogenesis, it is possible that the identification of compounds that influence *sel-12* activity will have implications for cancer, and possibly other human diseases.

35

**Implications of suppressor genes for drug testing.** Suppressor genes defined genetically, and candidates defined using the yeast two-hybrid system, encoding proteins of known biochemical

function will be useful for targeted drug design or the development of diagnostic tests for Alzheimer's disease or other diseases associated with alteration of members of the SEL-12/S182/STM2 family. For example, if a suppressor gene  
5 encodes a protein with an enzymatic activity, competitive or noncompetitive inhibitors of the enzyme might be effective drugs.

Suppressor genes encoding proteins of unknown biochemical  
10 function will also be useful for drug development. For example, the use of ribozymes based on suppressor genes, or the delivery via liposomes of vectors expressing suppressor genes, are potential therapeutic applications. The genetic analysis in *C. elegans* will provide a guide as to the nature of  
15 suppressor mutations. For example, a mutation that suppresses a *sel-12*(Alz) mutation that increases the activity of the suppressor gene would suggest the second strategy.

**Implications of suppressor genes for diagnostic tests.** The  
20 genetically-defined suppressor genes or candidate genes obtained using the yeast-two hybrid system will be used to identify human homologues. The cloned human homologues will be used to analyze pedigrees to see if mutations of the suppressor loci are associated with the development of Alzheimer's disease  
25 or other diseases. For example, the E5-1 gene was identified by using a cloned gene for pedigree analysis (Rogaev et al., 1995).

Suppressor genes may also be used as the basis for diagnostic  
30 tests. For example, mutations in suppressor genes implicated in Alzheimer's disease will be detected at the DNA level by Southern blotting or PCR/sequencing analysis, or at protein level, by Western blotting, immunoprecipitation or staining of cells or tissues.

35

**Antibodies for diagnosis.** Antisera to SEL-12 may cross-react with S182 and/or E5-1/STM2. Furthermore, peptides designed on the recognition of highly conserved regions, revealed by alignment of the predicted protein sequences of SEL-12, S182,

and E5-1/STM2, or of SEL-12, S182, E5-1/STM2, and SPE-4 (see Fig. 2), may be useful as diagnostic reagents. The conserved regions may reveal salient characteristics of a family of proteins, two of which have already been implicated in  
5 early-onset Alzheimer's disease. Such antisera could also be used to identify other members of the family, by screening expression libraries (Harlow and Lane, 1988)..

References of the Second Series of Experiments

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25

### Third Series of Experiments

#### Assessment of normal and mutant human presenilin function in *C. elegans*

5 Applicants provide evidence that normal human presenilins can substitute for *C. elegans* SEL-12 protein in functional assays in vivo. In addition, six familial Alzheimer's disease-linked mutant human presenilins were tested and found to have reduced ability to rescue the *sel-12* mutant phenotype, suggesting that  
10 they have lower than normal presenilin activity. A human presenilin 1 deletion variant that fails to be proteolytically processed and a mutant SEL-12 protein that lacks the carboxy terminus display considerable activity in this assay, suggesting that neither presenilin proteolysis nor the carboxy  
15 terminus is absolutely required for normal presenilin function. Applicants also show that *sel-12* is expressed in most neural and non-neural cell types in all developmental stages. The reduced activity of mutant presenilins together with as yet unknown gain-of-function properties may be a contributing  
20 factor in the development of Alzheimer's disease.

Genetic linkage studies have identified a number of genetic loci associated with familial Alzheimer's disease (1). Mutations in two genes, encoding the presenilins PS1 and PS2,  
25 are dominant and fully penetrant (1, 2, 3, 4, 5). PS1 and PS2 are related multipass transmembrane proteins that are about 67% identical in amino acid sequence. The presenilins are ubiquitously expressed (4, 5), and found in conjunction with intracellular membranes (6).

30

The normal function of presenilins, and the mechanism by which mutant presenilins cause Alzheimer's disease, are not yet known. The fact that more than thirty dominant, fully penetrant mutations in PS1 and PS2 are all missense mutations  
35 has suggested that Alzheimer's disease is associated with a gain-of-function activity of mutant proteins, although it remains formally possible that they partially lower activity of a dose-sensitive gene. Indeed, mutations may also have more than one effect on gene activity, and may have both



gain-of-function and loss-of-function characteristics. Classical studies have indicated that gain-of-function mutations in principle fall into one of three classes: hypermorphic mutations, which elevate gene activity; 5 antimorphic mutations, which reduce wild-type gene activity in trans (this category includes dominant-negative mutations); and neomorphic mutations, which create a novel activity (7). However, at the biochemical level, even the novel activity resulting from neomorphic mutations is related to the normal 10 mechanism of gene function. For example, neomorphic mutations in the *Drosophila* *awd* gene appear to alter the substrate specificity of nucleoside diphosphate kinase as well as reduce activity for its normal substrate (8), and mutations that cause 15 familial amyotrophic lateral sclerosis affect different activities of the normal protein, increasing the level of peroxidase activity (9) while in some cases reducing superoxide dismutase activity (10). Thus, an understanding of the normal function of presenilins as well as the nature of the dominant 20 mutations is crucial to elucidating the role of mutant presenilins in Alzheimer's disease.

Genetic studies in simple organisms offer a powerful approach to understanding the role of presenilins. A *C. elegans* gene, *sel-12*, encodes a protein that displays about 50% amino acid 25 sequence identity to PS1 and PS2 (11). *sel-12* was identified by reverting a phenotype caused by constitutive activation of LIN-12, a member of the LIN-12/Notch family of receptors [*sel* = *suppressor/enhancer of lin-12*]. Genetic analysis established that reducing or eliminating *sel-12* activity reduces the 30 activity of *lin-12*, and causes an egg-laying defective (Egl) phenotype. The Egl phenotype may be a direct consequence of reducing *lin-12* activity (12) or an independent effect of reducing *sel-12* activity. In this paper, applicants provide evidence that SEL-12 and the presenilins are functional 35 homologs, and that studies in *C. elegans* will be directly applicable to issues of presenilin structure and function in humans.

**MATERIALS AND METHODS**

**General methods and mutations used.** Methods for handling and culturing *C. elegans* have been described (13). The wild-type parent for all strains used was *C. elegans* var. Bristol strain N2 (13). *sel-12(ar131)* is described in ref. 11. All strains containing pLEX-based plasmids (see below) contained the *smg-1(r861)* and *unc-54(r293)* mutations (14). *smg-1* mutations stabilize mRNAs with long 3' untranslated regions (15), and *unc-54(r293)* is suppressed by *smg-1(r861)* (14). **pLEX-based constructs.** The pLEX vector has been described previously (16). It contains a 15.1 kb genomic region encompassing the *lin-12* gene, in which the normal translational start ATG was destroyed and replaced with a Not I site. cDNAs containing stop codons but lacking polyadenylation signals are inserted into the Not I site, and are efficiently expressed in a *smg-1* background. The following cDNAs were inserted into pLEX for this study.

***sel-12:*** The *sel-12* cDNA is described in ref. 11 and, as described below, results in efficient rescue of a *sel-12* mutant. Applicants note here that the *C. elegans* genome project has sequenced through the *sel-12* region (R. Waterston et al., personal communication). By comparing the genomic sequence with that of the available *sel-12* cDNA, applicants discovered that the cDNA has a frameshift mutation, beginning at codon 413, probably introduced by reverse transcription. This frameshift results in the substitution of 31 amino acids C-terminal to the frameshift mutation by 49 amino acids.

30

**PS1:** Full-length human PS1 cDNA and cDNA encoding the PS1 A246E substitution were generated by RT-PCR of cytoplasmic RNA isolated from skin fibroblasts of a patient harboring the A246E mutation (NIA Cell Repository #AG06848B) using a sense primer, **hAD3-ATG-Kpn** (GGGGTACCATGACAGAGTTACCTGCAC), and antisense primer, **hAD3-R-3'UTR** (CCGGGATCCATGGGATTCTAACCGC). PCR products were digested with Asp718 and BamHI and ~1.4 kb hPS1 cDNAs were gel purified and ligated to Bluescript KS+ vector (Stratagene, La Jolla, CA.) previously digested with Asp718 and BamHI, to

generate phPS1 and phPS1A246E. The cDNAs were sequenced in their entirety using a Sequenase kit (U.S. Biochemical Corp., Cleveland, OH).

- 5 To generate human PS1 cDNA encoding the M146L, H163R, L286V or C410Y substitutions (5), applicants used a four-way PCR strategy with two primer pairs and full-length PS1 cDNA as template. The inserts and junctions were sequenced using Sequenase (U.S. Biochemical Corp. (Cleveland, OH)).

10

- For M146L, primer pairs were hAD3-M146LF (GTCATTGTTGTCCTGACTATCCTCCTG)/hAD3-R284 (GAGGAGTAAATGAGAGCTGG) and hAD3-M146LR (CAGGAGGATAGTCAGGACAACAATGAC)/hAD3-237F (CAGGTGGTGGAGCAAGATG). PCR products from each reaction were  
15 gel purified, combined and subject to a second round of PCR with primers hAD3-237F and hAD3-R284. The resulting product was digested with KasI and PflMI and an ~300 bp gel purified fragment was ligated to KasI/PflMI-digested phPS1 to generate phPS1M146L. For H163R, primer pairs were hAD3-H163RF  
20 (CTAGGTCATCCGTGCCTGGC)/hAD3-R284 and hAD3-H163RR (GCCAGGCACGGATGACCTAG)/hAD3-237F. PCR products from each reaction were gel purified, combined and subject to a second round of PCR with primers hAD3-237F and hAD3-R284. The resulting products were digested with KasI and PflMI and a  
25 gel-purified ~300 bp fragment was ligated to KasI/PflMI-digested phPS1 to generate phPS1H163R.

- For L286V, primer pairs were hAD3-L286VF (CGCTTTTTTCCAGCTGTCATTTACTCC)/ hAD3-RL-GST  
30 (CCGGAATTCTCAGGTTGTGTTCCAGTC) and hAD3-L286VR (GGAGTAAATGACAGCTGGAAAAAGCG)/ hAD3-F146 (GGATCCATTGTTGTCATGACTATC). PCR products from each reaction were gel purified, combined and subject to a second round of PCR with primers hAD3-F146 and hAD3-RL-GST. The resulting  
35 products were digested with PflMI and BbsI and a gel purified ~480 bp fragment was ligated to PflMI/BbsI-digested phPS1 to generate phPS1L286V.

For C410Y, primer pairs were hAD3-C410YF

( C A A C C A T A G C C T A T T T C G T A G C C ) / L R T 7  
(GCCAGTGAATTGTAATACGACTCACTATAGGGC) and hAD3-C410YR  
(GGCTACGAAATAGGCTATGGTTG)/hAD3-243S (CCGGAATTCTGAATGGACTGCGTG).  
PCR products from each reaction were gel purified, combined and  
5 subject to a second round of PCR with primers hAD3-243S and  
LRT7. The resulting products were digested with BbsI and BamHI  
and an ~300 bp fragment was gel purified and ligated to  
BbsI/BamHI-digested phPS1 to generate phPS1C410Y.

- 10 The strategy for generating cDNA encoding hPS1 lacking exon 9  
(amino acids 290-319) was described previously (17).

PS2: Full-length cDNA encoding human PS2 was generated by  
RT-PCR of total human brain RNA using a sense primer,  
15 huAD4-ATGF (CCGGTACCAAGTGTTCTGTTGCTTCC) and antisense primer,  
hAD4-stopR (CCGTCTAGACCTCAGATGTAGAGCTGATG). PCR products were  
digested with Asp718 and XbaI and ~1.4 kB hPS2 cDNA were gel  
isolated and ligated to a vector fragment from expression  
plasmid pCB6 (17) previously digested with Asp718 and XbaI to  
20 generate phPS2. The insert was sequenced in its entirety using  
a Sequenase kit (U.S. Biochemical Corp., Cleveland, OH).

**Transgenic lines and rescue assays.** Transgenic lines were  
established by microinjection of plasmid mixtures into the  
25 hermaphrodite germline to create extrachromosomal arrays (18).  
By accepted convention, "Ex" is used to represent  
extrachromosomal arrays, and "Is" to represent integrated  
arrays (which can be generated from extrachromosomal arrays;  
see below).

30 pLEX and derivatives were injected at 20 µg/ml, 2 µg/ml or  
other concentrations (data not shown) into recipient strains of  
genotype *smg-1(r861) unc-54(r293); sel-12(ar131)* or *smg-1(r861)*  
*unc-54(r293)*. pRF4, a plasmid containing the cloned dominant  
35 *rol-6(sul1006)* gene (18) was used as a cotransformation marker  
and coinjected at a concentration of 100 µg/ml. F1 Roller  
progeny were picked, and F2 Roller progeny used to establish  
lines.

To assess rescue of *sel-12(ar131)*, approximately 40 L4 Rol progeny from at least three independent lines generated in a *smg-1(r861) unc-54(r293); sel-12(ar131)* background were picked individually and scored daily for the ability to lay eggs.

- 5 Applicants note here that rescue assays were performed using *sel-12(ar131)*, a strong partial loss-of-function allele of *sel-12*, because the strongest existing *sel-12* mutation, *sel-12(ar171)*, is somewhat suppressed by *smg-1* (data not shown). *sel-12(ar131)* displays variable penetrance (see Table
- 10 4) and expressivity. About 10% of *sel-12(ar131)* hermaphrodites have normal egg-laying, while 90% of hermaphrodites bloat with retained eggs; some of these bloated hermaphrodites never lay eggs, whereas others lay eggs. However, the proportion of hermaphrodites that lay eggs normally appears to be reduced by
- 15 the pLEX vector and/or the *rol-6* cotransformation marker (see Table 4). Applicants scored hermaphrodites as "Egl\*" only if they displayed robust egg-laying characteristic of wild-type hermaphrodites after two days as adults. However, applicants note that a greater proportion of hermaphrodites containing
- 20 human wild-type and mutant presenilins displayed improved egg-laying after one day compared to control hermaphrodites (data not shown), indicating that the criterion of normal egg-laying after two days underestimates rescuing activity. The pLEX vector causes a low level of sterility, and sterile
- 25 hermaphrodites were not scored.

Tabl 4

transgene	line	Egl*/total (%)
none	-	3/44 (6.8)
5 pLEX	1	1/71 (1.4)
	2	0/36 (0)
	3	1/40 (2.5)
SEL-12'	1	36/39 (92.3)
	2	38/40 (95.0)
	3	40/40 (100)
10 PS1	1	30/44 (68.1)
	2	33/40 (83.0)
	3	32/40 (80.0)
PS2	1	26/39 (67.0)
	2	33/40 (83.0)
	3	32/40 (80.0)
15 PS1 M146L	1	4/39 (10.3)
	2	6/37 (16.2)
	3	2/29 (6.9)
20 PS1 H163R	1	12/38 (31.6)
	2	7/38 (18.4)
	3	23/38 (60.5)
PS1 A286E	1	4/36 (11.1)
	2	5/39 (12.8)
	3	3/39 (7.7)
25 PS1 L266V	1	11/38 (28.9)
	2	6/38 (15.8)
	3	9/38 (23.7)
PS1 C410Y	1	7/36 (19.4)
	2	2/35 (5.7)
	3	7/38 (18.4)
30 PS1 ΔE9	1	26/39 (66.7)
	2	28/38 (73.7)
	3	17/27 (63.0)

35 Rescue of the *sel-12* egg-laying defective (Egl) and abnormal vulva phenotypes by normal and mutant human presenilins. The data is shown for transgenic lines generated by injecting the construct being tested at a concentration of 20 μg/ml. See  
40 Methods for details about generating and scoring transgenic lines.

\* Most PS1 mutations that cause Alzheimer's disease affect amino acids that are identical in SEL-12. The amino termini of PS1,  
45 PS2 and SEL-12 are not well conserved and are of different lengths. Therefore, for the mutations used here, the amino acid corresponding to M146 in PS1 is M115 in SEL-12; PS1 H163 is SEL-12 H132; PS1 A246 is SEL-12 V216; PS1 L286 is SEL-12 L255; PS1 C410 is SEL-12 C384. The ΔE9 mutation inhibits  
50 cleavage of PS1 (17); applicants note that SEL-12 is cleaved in a comparable position (Li and Greenwald, submitted).

' Egl' signifies robust egg-laying characteristic of wild-type hermaphrodites after two days as adults. This criterion is the  
55 most stringent applicants could apply, and underestimates the degree of rescuing activity (see Materials and Methods).

' Note that the *sel-12* cDNA used (11) has a frameshift mutation,

beginning at codon 413, resulting in the substitution of 31 amino acids C-terminal to the frameshift mutation by 49 amino acids (see Materials and Methods). See Materials and Methods for details about the human presenilin cDNAs.

5

Transgenic lines and  $\beta$ -galactosidase staining. pIB1Z17 [sel-12::lacZ] was made as follows: A unique BamHI site was inserted using the polymerase chain reaction at the second amino acid of a sel-12 rescuing genomic fragment containing 2.8 kb of 5' flanking region. A lacZ gene encoding a  $\beta$ -galactosidase protein containing a nuclear localization signal was excised from plasmid pPD16.43 (19) and inserted in frame into the BamHI site to generate the plasmid pIB1Z17. The predicted transcript contains an abnormally long 3' untranslated region, consisting of the sel-12 coding and 3' untranslated region, and is expected to be stabilized in a smg-1 background (15). pIB1Z17 was injected at a concentration of 10  $\mu$ g/ml into smg-1 unc-54 hermaphrodites. 9 independent lines containing extrachromosomal arrays were established. 4 independent attached lines were generated (using the method of C. Kari, A. Fire and R.K. Herman, personal communication) from one of the extrachromosomal arrays. All integrated and 7 of the 9 extrachromosomal arrays displayed staining; all staining lines had similar expression patterns, but some lines displayed more variability in intensity or penetrance of staining. The analysis described in this paper was performed using the attached array arIs17.

30

Mixed stage populations were grown at 25°, fixed using an acetone fixation protocol (20) and stained for  $\beta$ -galactosidase activity overnight at room temperature. Stained nuclei were identified based on their size, shape and position (21,22).

Counterstaining with 4,6-diamidino-2 phenylindole (DAPI) allowed visualization of all nuclei in the animal by fluorescence microscopy, facilitating the unambiguous identification of stained nuclei. Pictures of the staining pattern were taken at 1000X using TMAX400 (Kodak) film.

40

## RESULTS

A presenilin functional assay. There are currently no biochemical assays for presenilins, so there has been no direct assay for the effects of mutations on presenilin function. The high level of similarity between SEL-12, PS1 and PS2 suggested that the ability to rescue the distinctive egg-laying defective (Egl) phenotype caused by mutations that reduce or eliminate *sel-12* activity (11) could serve as an assay for presenilin function. The pLEX vector (16), which places inserted cDNAs under the control of *lin-12* regulatory sequences, can direct sufficient expression of a full-length *sel-12* cDNA (11; see Materials and Methods) to rescue the *sel-12(ar131)* Egl phenotype (Table 4). Applicants describe below how applicants have used this assay to evaluate the activity of normal and mutant human presenilins.

Rescue is assessed in transgenic lines, which are created by the microinjection of plasmid DNA into the hermaphrodite germline. This procedure generates extrachromosomal arrays, and there is some inherent variability in expression from different arrays, in part due to different numbers of copies of plasmid incorporated into the array (18). However, variability can be controlled for by examining multiple independent lines for each construct. Furthermore, arrays generated at the same concentration of injected DNA are likely to have comparable numbers of plasmid copies and therefore comparable levels of transgene expression (18). In all of the experiments described below, applicants have examined three independent lines for each construct, and compare the results for lines generated at the same concentration of injected DNA.

Rescue of a *sel-12* mutant by wild-type PS1 and PS2. Applicants have assessed the ability of wild-type human PS1 or PS2 cDNAs to rescue the Egl defect of *sel-12(ar131)* hermaphrodites (Table 4). Applicants found that the human proteins can efficiently substitute for SEL-12 in this assay, despite the vast evolutionary distance between nematodes and humans. The human proteins seem to be slightly less efficient than the *C. elegans* protein, but this small difference might in principle result



from inefficient translation of human presenilin RNA due to the different codon usage between *C. elegans* and humans, so that less presenilin protein may be produced even if a comparable level of mRNA is expressed from the extrachromosomal arrays.

5 The dramatic increase in *sel-12* activity when PS1 or PS2 is expressed using *lin-12* regulatory sequences, even at a relatively low concentration of injected DNA (Table 5), suggests that the human proteins are substituting for *C. elegans* SEL-12. An alternative interpretation is that the

10 human protein functions in this assay by stabilizing the mutant endogenous SEL-12(*arl31*) protein. However, this interpretation seems less likely in view of the efficient rescue; furthermore, a corrective interaction of this sort would imply that a SEL-12 and PS1 or PS2 complex is functional, which in itself would be

15 evidence for functional similarity of the *C. elegans* and human proteins.

**Activity of PS1 point mutants.** Applicants expressed five different human mutant PS1 proteins, each containing a single

20 amino acid alteration that causes Alzheimer's disease, and found that most displayed reduced ability to rescue *sel-12(arl31)* relative to wild-type PS1 (Table 4). These data suggest that the mutations that cause Alzheimer's disease may reduce but not eliminate normal presenilin activity. The

25 variable loss of extrachromosomal arrays confounds any determination of steady-state protein levels, so applicants do not know if the apparently lower activity of mutant presenilins results from reduced protein stability or reduced function.

30 **Activity of PS1  $\Delta$ E9.** PS1 is subject to endoproteolysis in vivo, and the PS1  $\Delta$ E9 mutant fails to be cleaved (17). Applicants have found that the human mutant PS1  $\Delta$ E9 retains a high level of activity, when arrays are formed at the concentration of 20  $\mu$ g/ml of injected DNA (Table 4). Since

35 arrays generated at a concentration of 20  $\mu$ g/ml of injected DNA are likely to contain many plasmid copies, which might mask a small difference in relative activity of PS1 and PS1  $\Delta$ E9, applicants generated arrays at the concentration of 2  $\mu$ g/ml of injected DNA. At this concentration of injected DNA, the

number of copies of plasmid present in the arrays should be reduced roughly tenfold (Mello et al., 1991). At this lower concentration, PS1  $\Delta$ E9 has reduced ability to rescue *sel-12(ar131)* as compared to wild-type PS1 (Table 5), suggesting that PS1  $\Delta$ E9, like the PS1 missense mutations, has reduced activity.

Table 5

transgene	line	Egl <sup>+</sup> /total (%)
pLEX	1	1/35 (2.9)
	2	0/38 (0)
SEL-12'	1	38/40 (95.0)
	2	40/40 (100)
	3	8/20 (40.0)
PS1	1	8/31 (25.8)
	2	36/41 (87.8)
	3	34/37 (92.0)
PS1 $\Delta$ E9	4	33/40 (91.9)
	5	34/40 (85.0)
	1	6/37 (16.2)
	2	5/39 (12.8)
	3	5/37 (13.5)
	4	14/41 (34.1)
	5	1/40 (2.5)

Rescue of the *sel-12* Egl phenotype by PS1 and PS1  $\Delta$ E9 expressed from arrays formed at a concentration of 2  $\mu$ g/ml. At 2  $\mu$ g/ml of injected DNA, expression from arrays or representation of the plasmid in the arrays may be reduced, accounting for the reduced activity of SEL-12 (transgenic line 3) and PS1 (transgenic line 1) compared to arrays generated at 20  $\mu$ g/ml (Table 4).

\* Egl<sup>+</sup>, see Table 4 legend and Materials and Methods.  
 ' see Table 4 legend and Materials and Methods for comments about the *sel-12* cDNA used.

40

**Examination of PS1 mutant transgenes in a *sel-12(+)* background.**  
 In an attempt to reveal gain-of-function activity, applicants assayed the ability of transgenes encoding mutant presenilins to cause phenotypes in a *sel-12(+)* background. Applicants saw no evidence for gain-of-function activity in this assay, as measured by the failure to obtain highly penetrant Egl or vulval abnormalities associated with abnormal *sel-12* or *lin-12* activity (data not shown). However, intrinsic limitations of the pLEX expression system (see Materials and Methods) may have

masked moderate changes in *sel-12* or *lin-12* activity, so a definitive assessment of the gain-of-function activity of mutant presenilins in *C. elegans* will not be possible until other expression systems or strategies are developed.

5

*sel-12* is widely expressed in neural and non-neural cells. Applicants have examined the expression pattern of transgenic lines carrying a *sel-12::lacZ* reporter gene (see Materials and Methods). Using this reporter gene, applicants have found that  
10 *sel-12*, like human presenilins (4, 5), is widely expressed in neural as well as non-neural cells (Fig. 3). Staining was seen in most cell types at all developmental stages from embryo to adult, with the notable exception of the intestine.

## 15 DISCUSSION

Sequence analysis revealed that SEL-12 is similar to human presenilins (11). Here, applicants have provided experimental evidence that SEL-12 is a bona fide presenilin, since it may be  
20 functionally replaced by either of the two human presenilins.

Applicants have also shown that *sel-12* is widely expressed in most neural and non-neural tissues of developing animals and adults. Furthermore, SEL-12 and PS1 also appear to have similar membrane topology (Doan et al., submitted; Li and  
25 Greenwald, submitted). These striking parallels between *C. elegans* and human presenilins suggest that studies of SEL-12 in *C. elegans* will bear directly on fundamental issues of presenilin structure and function. In the absence of any description of proteins similar to presenilins in single-celled  
30 organisms, including *Saccharomyces cerevisiae*, it appears that *C. elegans* is the simplest practical system for studying issues relevant to the biology of presenilins in vivo.

Since PS1 and PS2 appear to be similar in their ability to  
35 substitute for SEL-12, they may also have overlapping functions in mammals. As a consequence, studies of normal and mutant PS1 proteins should be directly applicable to PS2, and vice versa. Furthermore, since PS1 and PS2 have broad and overlapping expression patterns (4, 5), the phenotype of

mutants homozygous for null alleles of individual mouse presenilin genes may be less severe than the phenotype of double mutants, since there may be functional redundancy where the expression patterns overlap.

5 The rescue experiments also provide an indication that two regions of the presenilins are not essential for normal function. First, a SEL-12 protein lacking the last 31 amino acids is highly functional (see Table 4), suggesting that the  
10 C terminus is dispensable for SEL-12 function. Second, the PS1  $\Delta$ E9 protein, which 30 amino acids and fails to be proteolytically cleaved (17), retains considerable activity, suggesting that neither the deleted region nor cleavage is a prerequisite for presenilin activity. Applicants note that the  
15 rescue experiments do not address the possibility that the various mutations applicants tested have gain-of-function activity. Although the nature of the hypothetical gain-of-function activity of mutant presenilins is not clear, the mutant presenilins appear to increase the extracellular  
20 concentration of A $\beta$ 1-42(43) (ref. 23; Borchelt et al., submitted), and hence may cause Alzheimer's disease by fostering A $\beta$  deposition.

By expressing human genes in *C. elegans*, applicants have  
25 obtained evidence that six different presenilin mutations that cause early-onset Alzheimer's disease lower normal presenilin activity. Hypomorphic characteristics were manifested as reduced ability to rescue a *C. elegans* mutant defective in *sel-12* presenilin function. In the absence of any other assays  
30 for normal presenilin function, this information may be useful in considering the pathogenesis of Alzheimer's disease, and the development of mammalian models for the disease. It is possible that reduced presenilin activity may contribute to the development of Alzheimer's disease, either directly or in  
35 conjunction with an as yet unknown gain-of-function activity associated with mutant presenilins.

### Gain-of-function activity of *sel-12*(Alz) transgenes

The applicants have modified the *C. elegans sel-12* gene to  
 5 encode mutant proteins corresponding to PS1 mutants that cause  
 Alzheimer's disease in people. Transgenic *C. elegans* lines  
 containing these *sel-12*(Alz) genes have a novel  
 gain-of-function activity (manifested as an egg-laying  
 constitutive (Egl<sup>c</sup>) phenotype), which may be mechanistically  
 10 related to a gain-of-function activity that is presumed to  
 underlie the development of Alzheimer's disease. The  
 penetrance of the Egl<sup>c</sup> phenotype is enhanced in a *sel-12*(ar171)  
 background. An Egl<sup>c</sup> phenotype has been known to be associated  
 with stimulation of a G protein coupled serotonergic neural  
 15 pathway in *C. elegans* (Segalat et al., 1995; Mendel et al.,  
 1995; Koelle and Horvitz, 1996). The applicants are currently  
 exploring the effects of *sel-12*(Alz) mutations on other neural  
 signalling pathways that involve G protein coupled 7  
 transmembrane domain receptors, and neural signalling pathways  
 20 that may involve other kinds of signal transduction pathway.

#### *sel-12* mutant

	transgene	line	Egl <sup>c</sup> /Egl <sup>+</sup> (%)
25			
	+	1	0/37 (0)
		2	1/38 (2.6)
		3	0/38 (0)
30	H132R	1	2/38 (5.3)
		2	5/36 (13.9)
		3	2/39 (5.1)
	V216E	1	2/31 (6.5)
35	G363A	1	11/31 (35.5)
		2	13/40 (32.5)
		3	16/40 (40.0)

40 Data shown are for transgenes in a *sel-12*(ar171) genetic  
 background.

It may be that drugs that reduce serotonergic signalling or  
 45 other signalling pathways that the applicants will test will  
 suppress *sel-12*(Alz) gain-of-function phenotypes, thereby

suggesting potential prophylactic or therapeutic treatments, particularly if these signalling pathways or related pathways are shown to be affected in Alzheimer's disease. It may also be that the effect of drugs that reduce the gain-of-function activity of mutant presenilins will be potentiated by drugs that increase the normal activity of presenilins.

***spr* Genes: Suppressors of *sel-12(ar171)***

*sel-12(ar171)* hermaphrodites are egg-laying defective (Egl). The applicants have identified more than fifty extragenic suppressors of the Egl defect of *sel-12(ar171)* after EMS mutagenesis. The applicants have thus far assigned seven of the semidominant suppressor mutations to four new genes, named *spr-1* through *spr-4* [*spr* stands for suppressor of presenilin]. Two recessive suppressors probably define two additional *spr* genes. The remaining mutations are currently being analyzed and will be assigned to genes based on map position, genetic properties, and for recessive mutations, by complementation tests.

Gene dosage studies suggest that *spr-1V* mutations are hypermorphic, and that excess copies of the wild-type locus suppress *sel-12(ar171)*. The applicants are currently performing equivalent gene dosage studies with *spr-2 II*, which has been mapped to a 0.25 map unit interval corresponding to about 200 kb, and with *spr-3 III*. Meanwhile, assuming that the *spr-2* mutation is hypermorphic and that excess copies of the wild-type locus will suppress *sel-12(ar171)*, the applicants have embarked on cloning *spr-2* by injecting pools of cosmid clones from the *spr-2* region into *sel-12(ar171)*, and preliminary data suggest that this strategy will be successful.

The identification of suppressor mutations is a classical genetic tool used to identify other components of biochemical pathways. Extragenic suppressor mutations may identify new genes that are involved in presenilin-mediated processes, or reveal a functional connection between a previously known gene and presenilin function. Genetic and molecular

characterization of these "suppressor genes" in *C. elegans* will reveal the nature of their interactions with *sel-12* and *lin-12*. This analysis is directly relevant to Alzheimer's disease because the biochemical function of the presenilins is not  
5 known, so that a potential outcome of analyzing a suppressor gene would be an insight into the biochemistry of presenilin-mediated processes. If the suppressor gene has a known biochemical activity (based on sequence analysis), then, combined with the results of genetic analysis, the information  
10 will potentially be useful for the design and testing of therapeutic agents in both *C. elegans* and mammalian models, and ultimately for people. Furthermore, human homologs of the suppressor genes themselves may be useful diagnostic reagents, perhaps for the analysis of other inherited forms of  
15 Alzheimer's disease or for sporadic forms.

#### Topology and structure/function studies

The applicants have obtained evidence that SEL-12 presenilin  
20 contains 8 transmembrane domains (Li and Greenwald, submitted), and that certain regions of presenilins are dispensable for normal presenilin activity (Levitan et al., submitted). The applicants are continuing to do structure/function studies, by engineering mutant *sel-12* transgenes and assessing them in vivo  
25 in transgenic *C. elegans* lines for the ability to rescue defects associated with reducing *sel-12* activity and for gain-of-function activity.

Further structure/function studies in *C. elegans* may clarify  
30 the functions of domains of presenilin and be useful in conjunction with ultrastructural studies for rational drug design.

#### Gene and allele specificity studies

35

The applicants have been making double mutants between *sel-12(ar171)* and mutations in other secreted or transmembrane proteins. Thus far, a genetic interaction has been seen with a mutation in a TGF- $\beta$  receptor gene, *daf-1*. This result

suggests that *sel-12* may interact with genes other than *lin-12* and *glp-1*.

genotype	%Daf
5 <i>daf-1(m213)</i>	13%
<i>daf-1(m213); sel-12(ar171)</i>	98%

Interactions of this sort may enable the design of other  
10 suppressor/enhancer screens.

#### Other *C. elegans* presenilin genes

The applicants regularly search the *C. elegans* genomic sequence  
15 database for sequences related to *sel-12*. Recently, a  
predicted protein encoded by a sequence present on cosmid C18E3  
was found to have significant similarity to SEL-12. The  
applicants will test any potentially related sequences for the  
ability to complement *sel-12(ar131)* as described in Levitan et  
20 al. (submitted). Any sequences that behave like  
SEL-12/presenilins by this functional assay will be studied  
further.

Other *C. elegans* presenilins can be studied in the same way as  
25 *sel-12* in order to gain insights into presenilin structure and  
function, and Alzheimer's disease. The applicants will  
identify mutations in the new presenilins, identify suppressors  
of these new presenilin mutants, perform structure/function  
studies, and look for genetic interactions with *lin-12*, *glp-1*  
30 and other genes.



## References of Third Series of Experiments

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Greenwald, Iva  
Levitan, Diane
- (ii) TITLE OF INVENTION: IDENTIFICATION OF SEL-12 AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 9
- (iv) CORRESPONDENCE ADDRESS:
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  - (B) STREET: 1185 Avenue of the Americas
  - (C) CITY: New York
  - (D) STATE: New York
  - (E) COUNTRY: USA
  - (F) ZIP: 10036
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: White, John P.
  - (B) REGISTRATION NUMBER: 28,678
  - (C) REFERENCE/DOCKET NUMBER: 48231/JPW/AKC
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (212) 278-0400
  - (B) TELEFAX: (212) 391-0525

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 461 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES
- (ix) FEATURE:
  - (A) NAME/KEY: Protein
  - (B) LOCATION: 1..461
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  

Met	Pro	Ser	Thr	Arg	Arg	Gln	Gln	Glu	Gly	Gly	Gly	Ala	Asp	Ala	Glu
1				5					10					15	
Thr	His	Thr	Val	Tyr	Gly	Thr	Asn	Leu	Ile	Thr	Asn	Arg	Asn	Ser	Gln
			20				25					30			
Glu	Asp	Glu	Asn	Val	Val	Glu	Glu	Ala	Glu	Leu	Lys	Tyr	Gly	Ala	Ser

35 40 45  
 His Val Ile His Leu Phe Val Pro Val Ser Leu Cys Met Ala Leu Val  
 50 55 60  
 Val Phe Thr Met Asn Thr Ile Thr Phe Tyr Ser Gln Asn Asn Gly Arg  
 65 70 75 80  
 His Leu Leu Ser His Pro Phe Val Arg Glu Thr Asp Ser Ile Val Glu  
 85 90 95  
 Lys Gly Leu Met Ser Leu Gly Asn Ala Leu Val Met Leu Cys Val Val  
 100 105 110  
 Val Leu Met Thr Val Leu Leu Ile Val Phe Tyr Lys Tyr Lys Phe Tyr  
 115 120 125  
 Lys Leu Ile His Gly Trp Leu Ile Val Ser Ser Phe Leu Leu Leu Phe  
 130 135 140  
 Leu Phe Thr Thr Ile Tyr Val Gln Glu Val Leu Lys Ser Phe Asp Val  
 145 150 155 160  
 Ser Pro Ser Ala Leu Leu Val Leu Phe Gly Leu Gly Asn Tyr Gly Val  
 165 170 175  
 Leu Gly Met Met Cys Ile His Trp Lys Gly Pro Leu Arg Leu Gln Gln  
 180 185 190  
 Phe Tyr Leu Ile Thr Met Ser Ala Leu Met Ala Leu Val Phe Ile Lys  
 195 200 205  
 Tyr Leu Pro Glu Trp Thr Val Trp Phe Val Leu Phe Val Ile Ser Val  
 210 215 220  
 Trp Asp Leu Val Ala Val Leu Thr Pro Lys Gly Pro Leu Arg Tyr Leu  
 225 230 235 240  
 Val Glu Thr Ala Gln Glu Arg Asn Glu Pro Ile Phe Pro Ala Leu Ile  
 245 250 255  
 Tyr Ser Ser Gly Val Ile Tyr Pro Tyr Val Leu Val Thr Ala Val Glu  
 260 265 270  
 Asn Thr Thr Asp Pro Arg Glu Pro Thr Ser Ser Asp Ser Asn Thr Ser  
 275 280 285  
 Thr Ala Phe Pro Gly Glu Ala Ser Cys Ser Ser Glu Thr Pro Lys Arg  
 290 295 300  
 Pro Lys Val Lys Arg Ile Pro Gln Lys Val Gln Ile Glu Ser Asn Thr  
 305 310 315 320  
 Thr Ala Ser Thr Thr Gln Asn Ser Gly Val Arg Val Glu Arg Glu Leu  
 325 330 335  
 Ala Ala Glu Arg Pro Thr Val Gln Asp Ala Asn Phe His Arg His Glu  
 340 345 350  
 Glu Glu Glu Arg Gly Val Lys Leu Gly Leu Gly Asp Phe Ile Phe Tyr  
 355 360 365  
 Ser Val Leu Leu Gly Lys Ala Ser Ser Tyr Phe Asp Trp Asn Thr Thr  
 370 375 380  
 Ile Ala Cys Tyr Val Ala Ile Leu Ile Gly Leu Cys Phe Thr Leu Val  
 385 390 395 400  
 Leu Leu Ala Val Phe Lys Arg Ala Leu Pro Ala Leu Gln Phe Pro Phe  
 405 410 415

Ser Pro Asp Ser Phe Phe Thr Phe Val Pro Ala Gly Ser Ser Pro His  
 420 425 430  
 Leu Leu His Lys Ser Leu Lys Ser Val Tyr Tyr Ile Asn Ser Leu Phe  
 435 440 445  
 Leu Pro Phe Leu Cys Ile Ile Asn Phe Ser Ile Ile Ser  
 450 455 460

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 467 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

## (ix) FEATURE:

- (A) NAME/KEY: Active-site
- (B) LOCATION: 1..467

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Thr Glu Leu Pro Ala Pro Leu Ser Tyr Phe Gln Asn Ala Gln Met  
 1 5 10 15  
 Ser Glu Asp Asn His Leu Ser Asn Thr Val Arg Ser Gln Asn Asp Asn  
 20 25 30  
 Arg Glu Arg Gln Glu His Asn Asp Arg Arg Ser Leu Gly His Pro Glu  
 35 40 45  
 Pro Leu Ser Asn Gly Arg Pro Gln Gly Asn Ser Arg Gln Val Val Glu  
 50 55 60  
 Gln Asp Glu Glu Glu Asp Glu Glu Leu Thr Leu Lys Tyr Gly Ala Lys  
 65 70 75 80  
 His Val Ile Met Leu Phe Val Pro Val Thr Leu Cys Met Val Val Val  
 85 90 95  
 Val Ala Thr Ile Lys Ser Val Ser Phe Tyr Thr Arg Lys Asp Gly Gln  
 100 105 110  
 Leu Ile Tyr Thr Pro Phe Thr Glu Asp Thr Glu Thr Val Gly Gln Arg  
 115 120 125  
 Ala Leu His Ser Ile Leu Asn Ala Ala Ile Met Ile Ser Val Ile Val  
 130 135 140  
 Val Met Thr Ile Leu Leu Val Val Leu Tyr Lys Tyr Arg Cys Tyr Lys  
 145 150 155 160  
 Val Ile His Ala Trp Leu Ile Ile Ser Ser Leu Leu Leu Leu Phe Phe  
 165 170 175  
 Phe Ser Phe Ile Tyr Leu Gly Glu Val Phe Lys Thr Tyr Asn Val Ala  
 180 185 190  
 Val Asp Tyr Val Thr Val Ala Leu Leu Ile Trp Asn Phe Gly Val Val  
 195 200 205

Gly Met Ile Ser Ile His Trp Lys Gly Pro Leu Arg Leu Gln Gln Ala  
 210 215 220  
 Tyr Leu Ile Met Ile Ser Ala Leu Met Ala Leu Val Phe Ile Lys Tyr  
 225 230 235 240  
 Leu Pro Glu Trp Thr Ala Trp Leu Ile Leu Ala Val Ile Ser Val Tyr  
 245 250 255  
 Asp Leu Val Ala Val Leu Cys Pro Lys Gly Pro Leu Arg Met Leu Val  
 260 265 270  
 Glu Thr Ala Gln Glu Arg Asn Glu Thr Leu Phe Pro Ala Leu Ile Tyr  
 275 280 285  
 Ser Ser Thr Met Val Trp Leu Val Asn Met Ala Glu Gly Asp Pro Glu  
 290 295 300  
 Ala Gln Arg Arg Val Ser Lys Asn Ser Lys Tyr Asn Ala Glu Ser Thr  
 305 310 315 320  
 Glu Arg Glu Ser Gln Asp Thr Val Ala Glu Asn Asp Asp Gly Gly Phe  
 325 330 335  
 Ser Glu Glu Trp Glu Ala Gln Arg Asp Ser His Leu Gly Pro His Arg  
 340 345 350  
 Ser Thr Pro Glu Ser Arg Ala Ala Val Gln Glu Leu Ser Ser Ser Ile  
 355 360 365  
 Leu Ala Gly Glu Asp Pro Glu Glu Arg Gly Val Lys Leu Gly Leu Gly  
 370 375 380  
 Asp Phe Ile Phe Tyr Ser Val Leu Val Gly Lys Ala Ser Ala Thr Ala  
 385 390 395 400  
 Ser Gly Asp Trp Asn Thr Thr Ile Ala Cys Phe Val Ala Ile Leu Ile  
 405 410 415  
 Gly Leu Cys Leu Thr Leu Leu Leu Leu Ala Ile Phe Lys Lys Ala Leu  
 420 425 430  
 Pro Ala Leu Pro Ile Ser Ile Thr Phe Gly Leu Val Phe Tyr Phe Ala  
 435 440 445  
 Thr Asp Tyr Leu Val Gln Pro Phe Met Asp Gln Leu Ala Phe His Gln  
 450 455 460  
 Phe Tyr Ile  
 465

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 157 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

## (ix) FEATURE:

- (A) NAME/KEY: Active-site
- (B) LOCATION: 1..157

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Gly Lys Ser Pro Ser Asn Thr Glu Arg Xaa Val Ile Met Leu Phe  
 1 5 10 15  
 Val Pro Val Thr Leu Cys Met Ile Val Val Val Ala Thr Ile Lys Ser  
 20 25 30  
 Val Arg Phe Tyr Thr Glu Lys Asn Gly Gln Leu Ile Tyr Thr Pro Phe  
 35 40 45  
 Thr Glu Asp Thr Pro Ser Val Gly Gln Arg Leu Leu Asn Ser Val Leu  
 50 55 60  
 Asn Thr Leu Ile Met Ile Ser Val Ile Val Val Met Thr Ile Phe Leu  
 65 70 75 80  
 Val Val Leu Tyr Lys Tyr Arg Cys Tyr Lys Phe Ile His Gly Trp Leu  
 85 90 95  
 Ile Met Ser Ser Leu Met Leu Leu Phe Leu Phe Thr Tyr Ile Tyr Leu  
 100 105 110  
 Gly Glu Val Leu Lys Thr Tyr Asn Val Ala Met Asp Tyr Pro Thr Leu  
 115 120 125  
 Leu Leu Thr Val Trp Glu Leu Arg Gly Ser Gly His Gly Val His Pro  
 130 135 140  
 Leu Glu Gly Ala Phe Gly Ala Ala Glu Ala Tyr Leu Ser  
 145 150 155

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 465 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

- (ix) FEATURE:
- (A) NAME/KEY: Active-site
  - (B) LOCATION: 1..465

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Asp Thr Leu Arg Ser Ile Ser Ser Glu Leu Val Arg Ser Ser Gln  
 1 5 10 15  
 Leu Arg Trp Thr Leu Phe Ser Val Ile Ala Asn Met Ser Leu Thr Leu  
 20 25 30  
 Ser Ile Trp Ile Gly Val Tyr Asn Met Glu Val Asn Ser Glu Leu Ser  
 35 40 45  
 Lys Thr Tyr Phe Leu Asp Pro Ser Phe Glu Gln Thr Thr Gly Asn Leu  
 50 55 60  
 Leu Leu Asp Gly Phe Ile Asn Gly Val Gly Thr Ile Leu Val Leu Gly  
 65 70 75 80  
 Cys Val Ser Phe Ile Met Leu Ala Phe Val Leu Phe Asp Phe Arg Arg

BNSDOCID: <WD 97119564.1.1>



Gly  
465

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1500 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTTAATTAC CCAAGTTTGA GATGCCTTCC ACAAGGAGAC AACAGGAGGG CGGAGGTGCA	60
GATGCGGAAA CACATACCGT TTACGGTACA AATCTGATAA CAAATCGGAA TAGCCAAGAA	120
GACGAAAATG TTGTGGAAGA AGCGGAGCTG AAATACGGAG CATCTCACGT TATTCATCTA	180
TTTGTGCCGG TGTCACTATG CATGGCTCTG GTTGTTTTTA CGATGAACAC GATTACGTTT	240
TATAGTCAAA ACAATGGAAG GCATTTACTA TCACATCCTT TTGTCCGGA AACAGACAGT	300
ATCGTTGAGA AGGGATTGAT GTCACCTGGA AATGCTCTCG TCATGTTGTG CGTGGTTCGT	360
CTGATGACAG TTCTGCTGAT TGTTTTCTAT AAATACAAGT TTTATAAGCT TATTCATGGA	420
TGGCTTATTG TCAGCAGTTT TCTTCTTCTT TTCCTATTCA CTACAATCTA TGTGCAAGAA	480
GTTCTGAAAA GTTTCGATGT GTCTCCCAGC GCACTATTGG TTTGTGTTGG ACTGGGTAAC	540
TATGGAGTTC TCGGAATGAT GTGTATACAT TGGAAAGGTC CATTGCGTCT GCAACAGTTC	600
TACCTTATTA CAATGTCTGC ACTAATGGCT CTGGTCTTTA TCAAGTACCT ACCAGAATGG	660
ACTGTGTGGT TTGTGCTGTT TGTTATCTCG GTTTGGGATC TGTTTGCCGT GCTCACACCA	720
AAAGGACCAT TGAGATATTT GGTGGAAACT GCACAGGAGA GAAACGAGCC AATTTTCCCG	780
GCGCTGATTT ATTCGTCTGG AGTCATCTAT CCCTACGTTT TTGTTACTGC AGTTGAAAAC	840
ACGACAGACC CCCGTGAACC GACGTCGTCA GACTCAAATA CTTCTACAGC TTTTCCTGGA	900
GAGGCGAGTT GTTCATCTGA AACGCCAAAA CGGCCAAAAG TGAAACGAAT TCCTCAAAAA	960
GTGCAAATCG AATCGAATAC TACAGCTTCA ACGACACAAA ACTCTGGAGT AAGGGTGGAA	1020
CGGGAGCTAG CTGCTGAGAG ACCAACTGTA CAAGACGCCA ATTTTCACAG GCACGAAGAG	1080
GAAGAGAGAG GTGTGAAACT TGGTCTGGGC GACTTCATTT TCTACTCTGT TCTCCTCGGC	1140
AAGGCTTCAT CGTACTTTGA CTGGAACACG ACTATCGCTT GTTATGTGGC CATTCTTATC	1200
GGTCTCTGCT TCACTCTTGT CCTGCTCGCC GTCTTCAAAC GAGCACTCCC GGCTCTGCAA	1260
TTTCCATTTT CTCCGGACTC ATTTTITACT TTTGTACCCG CTGGATCATC ACCCCATTTG	1320
TTACACAAGT CTCTCAAAAG TGTTTATTAT ATTAATTCTC TGTTTTTGCC ATTTCTTTGC	1380
ATCATCAACT TTTGATTAT ATCTGAGCG ATCTCAAAGC TTTATTTTAC ATACCTATTT	1440

ATTTTGAAC TTTGTCATTT AAGTTATATA AATAATTTAT TAAAAAAAAA AAAAAAAAAA 1500

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 461 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

## (ix) FEATURE:

- (A) NAME/KEY: Active-site  
 (B) LOCATION: 1..461

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Pro	Ser	Thr	Arg	Arg	Gln	Gln	Glu	Gly	Gly	Gly	Ala	Asp	Ala	Glu	1	5	10	15
Thr	His	Thr	Val	Tyr	Gly	Thr	Asn	Leu	Ile	Thr	Asn	Arg	Asn	Ser	Gln	20	25	30	
Glu	Asp	Glu	Asn	Val	Val	Glu	Glu	Ala	Glu	Leu	Lys	Tyr	Gly	Ala	Ser	35	40	45	
His	Val	Ile	His	Leu	Phe	Val	Pro	Val	Ser	Leu	Cys	Met	Ala	Leu	Val	50	55	60	
Val	Phe	Thr	Met	Asn	Thr	Ile	Thr	Phe	Tyr	Ser	Gln	Asn	Asn	Gly	Arg	65	70	75	80
His	Leu	Leu	Ser	His	Pro	Phe	Val	Arg	Glu	Thr	Asp	Ser	Ile	Val	Glu	85	90	95	
Lys	Gly	Leu	Met	Ser	Leu	Gly	Asn	Ala	Leu	Val	Met	Leu	Cys	Val	Val	100	105	110	
Val	Leu	Met	Thr	Val	Leu	Leu	Ile	Val	Phe	Tyr	Lys	Tyr	Lys	Phe	Tyr	115	120	125	
Lys	Leu	Ile	His	Gly	Trp	Leu	Ile	Val	Ser	Ser	Phe	Leu	Leu	Leu	Phe	130	135	140	
Leu	Phe	Thr	Thr	Ile	Tyr	Val	Gln	Glu	Val	Leu	Lys	Ser	Phe	Asp	Val	145	150	155	160
Ser	Pro	Ser	Ala	Leu	Leu	Val	Leu	Phe	Gly	Leu	Gly	Asn	Tyr	Gly	Val	165	170	175	
Leu	Gly	Met	Met	Cys	Ile	His	Trp	Lys	Gly	Pro	Leu	Arg	Leu	Gln	Gln	180	185	190	
Phe	Tyr	Leu	Ile	Thr	Met	Ser	Ala	Leu	Met	Ala	Leu	Val	Phe	Ile	Lys	195	200	205	
Tyr	Leu	Pro	Glu	Trp	Thr	Val	Trp	Phe	Val	Leu	Phe	Val	Ile	Ser	Val	210	215	220	
Trp	Asp	Leu	Val	Ala	Val	Leu	Thr	Pro	Lys	Gly	Pro	Leu	Arg	Tyr	Leu	225	230	235	240

- 77 -

Val	Glu	Thr	Ala	Gln	Glu	Arg	Asn	Glu	Pro	Ile	Phe	Pro	Ala	Leu	Ile
				245					250					255	
Tyr	Ser	Ser	Gly	Val	Ile	Tyr	Pro	Tyr	Val	Leu	Val	Thr	Ala	Val	Glu
			260					265					270		
Asn	Thr	Thr	Asp	Pro	Arg	Glu	Pro	Thr	Ser	Ser	Asp	Ser	Asn	Thr	Ser
		275					280					285			
Thr	Ala	Phe	Pro	Gly	Glu	Ala	Ser	Cys	Ser	Ser	Glu	Thr	Pro	Lys	Arg
	290					295					300				
Pro	Lys	Val	Lys	Arg	Ile	Pro	Gln	Lys	Val	Gln	Ile	Glu	Ser	Asn	Thr
305					310					315					320
Thr	Ala	Ser	Thr	Thr	Gln	Asn	Ser	Gly	Val	Arg	Val	Glu	Arg	Glu	Leu
				325					330					335	
Ala	Ala	Glu	Arg	Pro	Thr	Val	Gln	Asp	Ala	Asn	Phe	His	Arg	His	Glu
			340					345					350		
Glu	Glu	Glu	Arg	Gly	Val	Lys	Leu	Gly	Leu	Gly	Asp	Phe	Ile	Phe	Tyr
		355					360					365			
Ser	Val	Leu	Leu	Gly	Lys	Ala	Ser	Ser	Tyr	Phe	Asp	Trp	Asn	Thr	Thr
	370					375					380				
Ile	Ala	Cys	Tyr	Val	Ala	Ile	Leu	Ile	Gly	Leu	Cys	Phe	Thr	Leu	Val
385					390					395					400
Leu	Leu	Ala	Val	Phe	Lys	Arg	Ala	Leu	Pro	Ala	Leu	Gln	Phe	Pro	Phe
				405					410					415	
Ser	Pro	Asp	Ser	Phe	Phe	Thr	Phe	Val	Pro	Ala	Gly	Ser	Ser	Pro	His
			420					425					430		
Leu	Leu	His	Lys	Ser	Leu	Lys	Ser	Val	Tyr	Tyr	Ile	Asn	Ser	Leu	Phe
		435					440					445			
Leu	Pro	Phe	Leu	Cys	Ile	Ile	Asn	Phe	Ser	Ile	Ile	Ser			
	450					455					460				

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGTCTGAGTT ACTAGTTTTC C

21

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 24 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: YES
- (iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGAATCTGAA GCACCTGTAA GCAT

24

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 448 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```

Met Leu Thr Phe Met Ala Ser Asp Ser Glu Glu Glu Val Cys Asp Glu
 1           5           10
Arg Thr Ser Leu Met Ser Ala Glu Ser Pro Thr Pro Arg Ser Cys Gln
          20           25           30
Glu Gly Arg Gln Gly Pro Glu Asp Gly Glu Asn Thr Ala Gln Trp Arg
          35           40           45
Ser Gln Glu Asn Glu Glu Asp Gly Glu Glu Asp Pro Asp Arg Tyr Val
          50           55           60
Cys Ser Gly Val Pro Gly Arg Pro Pro Gly Leu Glu Glu Glu Leu Thr
          65           70           75           80
Leu Lys Tyr Gly Ala Lys His Val Ile Met Leu Phe Val Pro Val Thr
          85           90           95
Leu Cys Met Ile Val Val Val Ala Thr Ile Lys Ser Val Arg Phe Tyr
          100          105          110
Thr Glu Lys Asn Gly Gln Leu Ile Tyr Thr Pro Phe Thr Glu Asp Thr
          115          120          125
Pro Ser Val Gly Gln Arg Leu Leu Asn Ser Val Leu Asn Thr Leu Ile
          130          135          140
Met Ile Ser Val Ile Val Val Met Thr Ile Phe Leu Val Val Leu Tyr
          145          150          155          160
Lys Tyr Arg Cys Tyr Lys Phe Ile His Gly Trp Leu Ile Met Ser Ser
          165          170          175
Leu Met Leu Leu Phe Leu Phe Thr Tyr Ile Tyr Leu Gly Glu Val Leu
          180          185          190
Lys Thr Tyr Asn Val Ala Met Asp Tyr Pro Thr Leu Leu Leu Thr Val
          195          200          205

```

Trp Asn Phe Gly Ala Val Gly Met Val Cys Ile His Trp Lys Gly Pro  
 210 215 220  
 Leu Val Leu Gln Gln Ala Tyr Leu Ile Met Ile Ser Ala Leu Met Ala  
 225 230 235 240  
 Leu Val Phe Ile Lys Tyr Leu Pro Glu Trp Ser Ala Trp Val Ile Leu  
 245 250 255  
 Gly Ala Ile Ser Val Tyr Asp Leu Val Ala Val Leu Cys Pro Lys Gly  
 260 265 270  
 Pro Leu Arg Met Leu Val Glu Thr Ala Gln Glu Arg Asn Glu Pro Ile  
 275 280 285  
 Phe Pro Ala Leu Ile Tyr Ser Ser Ala Met Val Trp Thr Val Gly Met  
 290 295 300  
 Ala Lys Leu Asp Pro Ser Ser Gln Gly Ala Leu Gln Leu Pro Tyr Asp  
 305 310 315 320  
 Pro Glu Met Glu Glu Asp Ser Tyr Asp Ser Phe Gly Glu Pro Ser Tyr  
 325 330 335  
 Pro Glu Val Phe Glu Pro Pro Leu Thr Gly Tyr Pro Gly Glu Glu Leu  
 340 345 350  
 Glu Glu Glu Glu Glu Arg Gly Val Lys Leu Gly Leu Gly Asp Phe Ile  
 355 360 365  
 Phe Tyr Ser Val Leu Val Gly Lys Ala Ala Ala Thr Gly Ser Gly Asp  
 370 375 380  
 Trp Asn Thr Thr Leu Ala Cys Phe Val Ala Ile Leu Ile Gly Leu Cys  
 385 390 395 400  
 Leu Thr Leu Leu Leu Leu Ala Val Phe Lys Lys Ala Leu Pro Ala Leu  
 405 410 415  
 Pro Ile Ser Thr Thr Phe Gly Leu Ile Phe Tyr Phe Ser Thr Asp Asn  
 420 425 430  
 Leu Val Arg Pro Phe Met Asp Thr Leu Ala Ser His Gln Leu Tyr Ile  
 435 440 445

What is claim d is:

1. An isolated nucleic acid molecule encoding a SEL-12.
- 5 2. An isolated nucleic acid molecule encoding a mutated SEL-12.
3. An isolated nucleic acid molecule of claim 2, wherein the mutated SEL-12 contains at least one of the following:  
10 leucine at position 115, arginine at position 132, glutamic acid at position 215, valine at position 229, valine at position 254, valine at position 255, valine at position 371, tyrosine at position 387, isoleucine at position 104 or valine at position 204.
- 15 4. An isolated nucleic acid molecule of claim 2, wherein the mutated SEL-12 contains one or more alterations.
5. An isolated nucleic acid molecule encoding a  
20 *Caenorhabditis elegans* protein that is homologous to SEL-12.
6. An isolated DNA molecule of claim 2 or 3, wherein the mutation is generated by in vitro mutagenesis.
- 25 7. An isolated DNA molecule of any of claim 1 to 6.
8. An isolated cDNA molecule of claim 7.
- 30 9. An isolated genomic DNA molecule of claim 7.
10. An isolated RNA molecule of any of claim 1 to 6.
11. An isolated nucleic acid molecule of claim 1, wherein the  
35 SEL-12 has substantially the same amino acid sequence as the amino acid sequence shown in Figure 1A.
12. A nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a unique sequence within

the sequence of a nucleic acid molecule of claim 1.

13. A DNA molecule of claim 12.
- 5 14. An RNA molecule of claim 12.
15. A vector which comprises the isolated nucleic acid molecule of claim 1.
- 10 16. An isolated nucleic acid molecule of claim 7, 8 or 9 operatively linked to a promoter of RNA transcription.
17. The vector of claim 15 or 16, wherein the vector is a plasmid.
- 15 18. The plasmid of claim 17 designated pMX8 (ATCC Accession No. 97278).
19. The plasmid of claim 17 designated p1-1E (ATCC Accession  
20 No. 97279).
20. A host vector system for the production of a SEL-12 protein which comprises the vector of claim 15 and a suitable host.
- 25 21. A host vector system of claim 20, wherein the suitable host is a bacterial cell, insect cell, plant or mammalian cell.
- 30 22. A purified SEL-12 protein or a fragment thereof.
23. A purified mutated SEL-12 protein or a fragment thereof.
24. A method for production of an antibody comprising:  
35 a) administering an amount of the purified protein or fragment of SEL-12 or mutated SEL-12 to a suitable animal effective to produce an antibody against SEL-12 or mutated SEL-12 protein in the animal; and  
b) recovering the produced antibody so produced from the

animal.

25. A method for production of an antibody capable of binding to wild-type or mutant S182 or E5-1/STM2, wherein the antibody is produced by in vitro immunization.
26. A method for production of an antibody capable of binding to wild-type or mutant S182 or E5-1/STM2, wherein the antibody is produced by screening a differential phage display library.
27. A method for production of an antibody capable of binding to wild-type or mutant S182 or E5-1/STM2 comprising:
- a) determining conserved regions revealed by alignment of the SEL-12, S182 and E5-1/STM2 protein sequences;
  - b) synthesizing peptides corresponding to the revealed conserved regions;
  - c) administering an amount of the synthesized peptides to a suitable animal effective to produce an antibody against the peptides in the animal; and
  - b) recovering the produced antibody so produced from the animal.
28. An antibody produced by the method of any of claim 24 to 27.
29. A monoclonal antibody of claim 28.
30. A transgenic animal comprising a DNA molecule of any of claims 7 to 9.
31. The transgenic animal of claim 30 wherein the animal is a *Caenorhabditis elegans*.
32. A transgenic *Caenorhabditis elegans* animal comprising wild-type or mutant human S182 gene.
33. A transgenic *Caenorhabditis elegans* animal comprising wild-type or mutant human STM2/E5-1 gene.



34. A transgenic *Caenorhabditis elegans* animal comprising wild-type or mutant human presenilin gene.
- 5 35. A transgenic *Caenorhabditis elegans* animal of any of claim 30-34, wherein the wild-type or mutant human S182, or wild-type or mutant STM2/E5-1 gene, or mutant human presenilin gene is under the control of *sel-12* or *lin-12* regulatory sequence.
- 10 36. A transgenic *Caenorhabditis elegans* animal of claim 30-34, wherein the wild-type or mutant human S182, or wild-type or mutant STM2/E5-1 gene, or mutant human presenilin gene is under the control of a regulatory sequence other than the *sel-12* or *lin-12* regulatory sequence.
- 15 37. A transgenic *Caenorhabditis elegans* animal of claim 30-36 having an egg-laying constitutive (*Egl<sup>c</sup>*) phenotype.
- 20 38. A transgenic *Caenorhabditis elegans* animal of claim 30-36 having a phenotype other than egg-laying constitutive (*Egl<sup>c</sup>*).
- 25 39. A transgenic *Caenorhabditis elegans* animal having a *sel-12* allele that reduces, eliminates or elevates *sel-12* activity.
- 30 40. A transgenic *Caenorhabditis elegans* animal having a *sel-12* transgene carrying a mutation that is equivalent to a mutation that causes Alzheimer's disease [*sel-12(Alz)*].
- 35 41. A method for identifying a compound which is capable of ameliorating Alzheimer disease comprising administering effective amount of the compound to the transgenic animal of any of claim 30-40, the alteration of the conditions of the transgenic animal indicating the compound is capable of ameliorating Alzheimer's disease.
42. A method of claim 41, wherein at least one signalling pathway is altered.

43. A method of claim 42, wherein the signalling pathway is a neuronal signalling pathway.
- 5 44. A method of claim 43, wherein the signalling pathway is the serotonergic signalling pathway.
45. A previously unknown compound identified by the method of any of claim 41-44.
- 10 46. A pharmaceutical composition comprising an effective amount of the compound identified by the method of claim of any of 41-44 and a pharmaceutically acceptable carrier.
- 15 47. A method for determining whether a compound is capable of ameliorating Alzheimer's disease comprising:
- a) treating *Caenorhabditis elegans* mutants having reduced, increased or altered *sel-12* activity with the compound; and
- 20 b) determining whether the compound suppresses, enhances or has no effect on the phenotype of the mutant, the suppression or enhancement of the phenotype indicating that the compound is capable of ameliorating Alzheimer's disease.
- 25 48. A method of claim 47, wherein the *Caenorhabditis elegans* mutant to be treated is *sel-12(ar171)* (ATCC Accession No. 97292).
- 30 49. A method of claim 47, wherein the *Caenorhabditis elegans* mutant to be treated is *sel-12(ar131)* (ATCC Accession No. 97293).
50. A method of claim 47, wherein the *Caenorhabditis elegans* mutant to be treated is a *sel-12* allele that reduces or eliminates *sel-12* activity.
- 35 51. A method of claim 47, wherein the *Caenorhabditis elegans* mutant to be treated is a *sel-12* allele that elevates or alters *sel-12* activity.

52. A method of claim 47, wherein the *Caenorhabditis elegans* mutant to be treated is a *sel-12* transgenic animal carrying a mutation in *sel-12* that is equivalent to a mutation that causes Alzheimer's disease [*sel-12*(Alz)].
53. A method of claim 47, wherein the *Caenorhabditis elegans* mutant to be treated is a *sel-12* transgenic animal carrying a mutation in *sel-12*, and results in an *Egl<sup>c</sup>* phenotype.
54. A method of claim 47, wherein the *Caenorhabditis elegans* mutant to be treated is a *sel-12* transgenic animal carrying a mutation in *sel-12* that is equivalent to a mutation that causes Alzheimer's disease, and results in a phenotype other than *Egl<sup>c</sup>* phenotype.
55. A method of claim 47, wherein the *Caenorhabditis elegans* mutant to be treated is a transgenic animal from any of claim 30-40.
56. A previously unknown compound determined by the method of any of claim 47-55 to be capable of ameliorating Alzheimer's disease.
57. A pharmaceutical composition comprising an effective amount of the compound determined by the method of claim 47-55 to be capable of ameliorating Alzheimer's disease and a pharmaceutically acceptable carrier.
58. A method for identifying a suppressor of the multivulva phenotype of *lin-12* gain-of-function mutation comprising:
- a) mutagenizing *lin-12* *Caenorhabditis elegans* worms with an effective amount of an appropriate mutagen;
  - b) screening for revertants in the F1, F2 and F3 generations; and
  - c) isolating the screened revertant, thereby identifying a suppressor of the multivulva phenotype of *lin-12* gain-of-function mutation.

59. A suppressor identified by method of claim 58.
60. An animal having a suppressor of claim 59, designated *sel-12(ar131)* (ATCC Accession No. 97293).
- 5 61. An animal having a suppressor of claim 59, designated *sel-12(ar133)*.
62. A method for identifying a mutant *sel-12* gene which  
10 reduces *sel-12* function comprising:  
a) mutagenizing *Caenorhabditis elegans* worms with an effective amount of an appropriate mutagen;  
b) performing complementation screening of the  
15 mutagenized worms to determine if a descendant of a mutagenized worm bears a mutation that fails to complement a suppressor of claim 59 for the *Egl* defect; and  
c) isolating the individual worm and determining the  
20 phenotype of worms carrying the new allele in its homozygous form and in trans to a deficiency, thereby identifying a mutant *sel-12* gene which reduces *sel-12* function.
63. A method for identifying a mutant *sel-12* gene which  
25 reduces or elevates *sel-12* function comprising:  
a) mutagenizing *Caenorhabditis elegans* worms with an effective amount of an appropriate mutagen;  
b) identifying suppressors or enhancers of *daf-1* single  
30 mutants, or *daf-1; sel-12* double mutants, or mutations in other genes that interact with *sel-12*;  
c) isolating the individual worm and determining the phenotype of worms carrying the new allele in its homozygous form and in trans to a deficiency, thereby  
35 identifying a mutant *sel-12* gene which reduces *sel-12* function.
64. A method of claim 63, further comprising performing DNA sequence analysis of the identified mutant *sel-12* gene to determine the molecular lesion responsible for the

mutation.

65. A mutant *sel-12* gene identified by the method of any of claim 62-64.
- 5 66. An animal having a mutant *sel-12* gene of claim 62, designated *sel-12 (ar171)* (ATCC Accession No. 97292).
67. A method for producing extragenic suppressors or enhancers of a *sel-12* allele comprising:
- 10 a) mutagenizing *sel-12* mutant hermaphrodites with an effective amount of a mutagen;
- b) screening for revertants in the F1, F2 and F3 generations; and
- 15 c) isolating the screened revertant, thereby producing extragenic suppressors or enhancers of a *sel-12* allele.
68. A method for producing extragenic suppressors of a *sel-12* allele comprising:
- 20 a) mutagenizing *sel-12(ar171)* or *sel-12(ar131)* mutant hermaphrodites with an effective amount of a mutagen;
- b) screening for revertants in the F1, F2 and F3 generations; and
- 25 c) isolating the screened revertant, thereby producing extragenic suppressors or enhancers of a *sel-12* allele.
69. A method for producing extragenic suppressors or enhancers of a *sel-12* allele comprising:
- 30 a) mutagenizing *daf-1(m213); sel-12(ar171)* mutant hermaphrodites with an effective amount of a mutagen;
- b) screening for revertants in the F1, F2 and F3 generations; and
- 35 c) isolating the screened revertant, thereby producing extragenic suppressors or enhancers of a *sel-12* allele.
70. A method for producing extragenic suppressors or enhancers

of a *sel-12*(Alz) mutant comprising:

- a) mutagenizing *sel-12* (Alz) hermaphrodites with an effective amount of a mutagen;
- b) screening for revertants in the F1, F2 and F3 generations; and
- c) isolating the screened revertant, thereby producing extragenic suppressors or enhancers of a *sel-12*(Alz) mutant.

71. A suppressor or enhancers produced by the method of any of claim 67-70.

72. A suppressor of presenilin, designated *spr-1*, *spr-2*, *spr-3* or *spr-4*.

73. The human homolog of *spr-1*, *spr-2*, *spr-3* or *spr-4*.

74. A human homolog of a gene defined by extragenic suppressor or enhancer of a *sel-12* mutant.

75. A *Drosophila* homolog of a gene defined by extragenic suppressors of a *sel-12* mutant.

76. A mouse homolog of a gene defined by extragenic suppressor of a *sel-12* mutant.

77. The homolog of any of claim 73-76, wherein the *sel-12* mutant is *sel-12*(ar171) (ATCC Accession No. 97292).

78. The homolog of any of claim 73-76, wherein the *sel-12* mutant is *sel-12*(Alz) transgene.

79. The homolog of any of claim 73-76, wherein the *sel-12* mutant is *sel-12*(ar131) (ATCC Accession No. 97293).

80. The homolog of any of claim 73-76, wherein the *sel-12* mutant is any other *sel-12* allele.

81. A method for identifying a suppressor gene comprising

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/15727

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-21, 65

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/15727

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

536 / 23.5, 24.31, 24.33, 25.1

435 / 320.1, 252.3

## B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

536 / 23.5, 24.31, 24.33, 25.1

435 / 320.1, 252.3

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Automated Patent System - USPAT, JPOABS, EPOABS

DIALOG FILES - 5, 11, 73, 76, 144, 155, 156, 185, 434, 440, 444, and 636

Search Terms - sel-12, Caenorhabditis elegans, suppress, enhance, ar131, ar133, and ar171



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/15727

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database dbEST, Release 072795, 27 July 1995, National Center for Biothechnology Information, National Library of Medicine, National Institutes of Health, GenBank Accession number H19012, Name H19012, ENTREZ Document Retrieval System, Release 18.0, 15 August 1995, see entire document.	12, 13
-		_____
Y		14, 15

## INTERNATIONAL SEARCH REPORT

International application N.  
PCT/US96/15727**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C07H 21/04, 21/02; C12N 15/12, 15/70, 1/21

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LEVITAN et al. Facilitation of lin-12 -mediated signalling by sel-12, a Caenorhabditis elegans S182 Alzheimer's disease gene. Nature. 28 September 1995, Vol. 377, pages 351-354, see entire document.	1, 2, 4-17, 20, 21, and 65
Y		3, 18, and 19
Y	STRATAGENE CLONING SYSTEMS CATALOG, issued 1993, La Jolla, CA pages 27, 31,2 and 313, see entire document.	18 and 19
Y	SUNDARAM et al. Suppressors of a lin-12 Hypomorph Define Genes That Interact With Both lin-12 and glp-1 in Caenorhabditis elegans. Genetics. November 1993, Vol. 135, pages 765-783, see entire document.	1-21 and 65

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	A	document member of the same patent family
*O* documents referring to an oral disclosure, use, exhibition or other means		
*P* documents published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

18 DECEMBER 1996

Date of mailing of the international search report

21 JAN 1997

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
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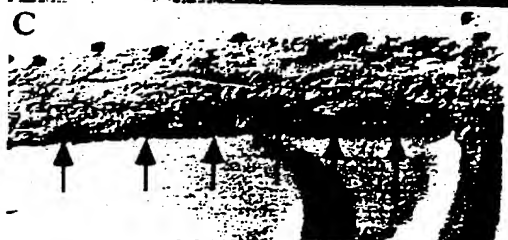
Authorized officer

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Telephone No. (703) 308-0196

**FIGURE 3A**

**FIGURE 3B**



**FIGURE 3C**

**FIGURE 3D**

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FIGURE 2B

TM7				
-----				
XX				
SEL-12	EPIFPALIYS	SGVIYPYVLV	TAVENTTDP	EPTSSDSNTS
S182	ETLFPALIYS	STMVW...LV	NMAEGDPEAQ	RRVSKNSKYN
E5-1	EPIFPALIYS	SAMVW...TV	GMAKLDP...	...SSQ GALQ
SPE-4	KCVLNLIMFS	ANEKRLTAGS	NQEETNEGEE	STIRRTVKQT
				IEYYTKREAQ
SEL-12	SE.....	.....	.....	..TPKRPKVK
S182	T.....	.....	.....	.....VA
E5-1	S.....	.....	.....	.....YD
SPE-4	DDEFYQKIRQ	RRAAINPDSV	PTEHSPLVEA	EPSPIELKEK
				NSTEELSDDE
SEL-12	NTTASTTQNS	GVRVERELAA	ERPTVQDANF	HRHEEEERG.
S182	WEAQRDSHLG	PHRSTPE SRA	AVQELSSSIL	AGEDPEERG.
E5-1	FEPPLTGYPG	EEL.....	.....	..EEEEERG.
SPE-4	SDTSETSSGS	SNLSSSDSST	TVSTSDISTA	EECDQKEWDD
				LVSNSLPNND
X				
SEL-12	.....	.....VKLGL	GDFIFYSVLL	GKASSYF..D
S182	.....	.....VKLGL	GDFIFYSVLV	GKASATASGD
E5-1	.....	.....VKLGL	GDFIFYSVLV	GKAAATGSGD
SPE-4	KRPATAADAL	NDGEVLR LGF	GDFVIFYSLLI	GQAAASGCP.
				.FAVISAALG
TM8				
-----				
SEL-12	ILIGLCFTLV	LLAVFKRALP	ALQFPFSPDS	FFTFFVPAGSS
S182	ILIGLCFTLL	LLAIFKKALP	ALPISITFGL	VFFYFATDYL
E5-1	ILIGLCFTLL	LLAVFKKALP	ALPISTTFGL	IFYFSTDNLV
SPE-4	ILFGLVVTLT	VFSTEESTTP	ALPLPVICGT	FCYFSSMFFW
				EQLYG.....
TM9?				
-----				
SEL-12	VYVINSFLFP	FLCIINFSII	S	
S182	QFYI.....	.....		
E5-1	QLYI.....	.....		

3/5

## FIGURE 2A

SEL-12 ..... ..MPSTRRQQ  
 S182 .....MTEIP APLSYFQNAQ MSEDNHLST VRSQNDNRER QEH.NDRRSL  
 E5-1 MLTFMADSE EEVCDERTSL MSAESPTPRS CQEGRQGPED GENTAQRWSQ

SEL-12 EGGGADAETH TVYGTNLITN RNSQEDENVV EEAELKYGAS HVIHLFVPVS  
 S182 GHPEPLSNGR PQGNSRQVVE QDEED.... EELTLKYGAK HVIMLFVPVT  
 E5-1 ENEEDGEEDP DRYVCSGVP G RPPGLE.... EELTLKYGAK HVIMLFVPVT  
 SPE-4 ..... ..MDTLRSI SSELVRSSQL RWTLFVSIAN

## TM1

SEL-12 LCMALVV.FT MNTITFYSQN NGRHLLSHPF VRETDSIVEK GLMSLGNALV  
 S182 LCMVVVV.AT IKSVSFYTRK DG.QLIYTPF TEDTETVGQR ALHSILNAAI  
 E5-1 LCMIVVV.AT IKSVRFYTEK NG.QLIYTPF TEDTPSVGQR LNSVLNTLI  
 SPE-4 MSLLSIWIG VYNMEVNSEL SKTYFLDPSF EQTIGNL... LLDGFINGVG

## TM2

## TM3

SEL-12 MLCVVV.LMTV LLIVFYKYKF YKLIHGWLIV SSLLLLF... LFTT  
 S182 MISVIVVMTI LLVVLKYRC YKVIHAWLII SSLLLLF... FFSF  
 E5-1 MISVIVVMTI FLVVLKYRC YKFIHGWLIM SSLMLLF... LFTY  
 SPE-4 TILVLGCVSF IMLAFVLFDF RRIVKAWLTL SCLLILFGVS AQTLDHDMFSQ

## TM4

SEL-12 IYVQEVLSKF DVSPSALLVL FGLGNYGVLG MNCIHWKGPL RLQQFYLIITM  
 S182 IYLGVEVFKTY NVAVDYVTVA LLIWNFQVVG MISIHWKGPL RLQQAYLITMI  
 E5-1 IYLGVEVFKTY NVAMDYPTLL LTVWNFGAVG MNCIHWKGPL VLQQAYLITMI  
 SPE-4 VFDQDDNNQY YMTIVLIVVP TVVYGFG..G IYAFFSNSSL ILHQIFVVIN

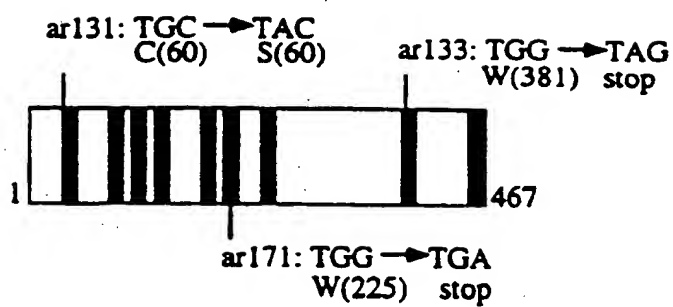
## TM5

## TM6

SEL-12 SALMALVFIK YLPEWTVWFV LFVISVVDLV AVLTPKGPLR YLVETAQERN  
 S182 SALMALVFIK YLPEWTAALI LAVISVVDLV AVLCPKGPLR MLVETAQERN  
 E5-1 SALMALVFIK YLPEWSAWVI LGAISVVDLV AVLCPKGPLR MLVETAQERN  
 SPE-4 CSLISVFYLR VFPSKTTWFFV LWIVLFWDLF AVLAPMGPLK KVQEKASDYS

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FIGURE 1B





performing DNA sequence analysis of the suppressor of claim 68 to identify the suppressor gene.

82. The suppressor gene identified by method of claim 81.
- 5 83. A human suppressor gene of claim 82.
84. A *Drosophila* suppressor gene of claim 82.
- 10 85. A mouse suppressor gene of claim 82.
86. The method of any of claim 59, 60, 61, 65, 66 or 67, wherein the mutagen is ethyl methanesulfonate.